

University of Groningen

Interleukin-1 beta Attenuates Myofibroblast Formation and Extracellular Matrix Production in Dermal and Lung Fibroblasts Exposed to Transforming Growth Factor-beta 1

Mia, Masum M.; Boersema, Miriam; Bank, Ruud A.

Published in:
 PLoS ONE

DOI:
[10.1371/journal.pone.0091559](https://doi.org/10.1371/journal.pone.0091559)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
 Publisher's PDF, also known as Version of record

Publication date:
 2014

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Mia, M. M., Boersema, M., & Bank, R. A. (2014). Interleukin-1 beta Attenuates Myofibroblast Formation and Extracellular Matrix Production in Dermal and Lung Fibroblasts Exposed to Transforming Growth Factor-beta 1. PLoS ONE, 9(3), [e91559]. <https://doi.org/10.1371/journal.pone.0091559>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Interleukin-1 β Attenuates Myofibroblast Formation and Extracellular Matrix Production in Dermal and Lung Fibroblasts Exposed to Transforming Growth Factor- β 1

Masum M. Mia, Miriam Boersema, Ruud A. Bank*

Division of Medical Biology, Department of Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands,

Abstract

One of the most potent pro-fibrotic cytokines is transforming growth factor (TGF β). TGF β is involved in the activation of fibroblasts into myofibroblasts, resulting in the hallmark of fibrosis: the pathological accumulation of collagen. Interleukin-1 β (IL1 β) can influence the severity of fibrosis, however much less is known about the direct effects on fibroblasts. Using lung and dermal fibroblasts, we have investigated the effects of IL1 β , TGF β 1, and IL1 β in combination with TGF β 1 on myofibroblast formation, collagen synthesis and collagen modification (including prolyl hydroxylase, lysyl hydroxylase and lysyl oxidase), and matrix metalloproteinases (MMPs). We found that IL1 β alone has no obvious pro-fibrotic effect on fibroblasts. However, IL1 β is able to inhibit the TGF β 1-induced myofibroblast formation as well as collagen synthesis. Glioma-associated oncogene homolog 1 (GLI1), the Hedgehog transcription factor that is involved in the transformation of fibroblasts into myofibroblasts is upregulated by TGF β 1. The addition of IL1 β reduced the expression of GLI1 and thereby also indirectly inhibits myofibroblast formation. Other potentially anti-fibrotic effects of IL1 β that were observed are the increased levels of MMP1, -2, -9 and -14 produced by fibroblasts exposed to TGF β 1/IL1 β in comparison with fibroblasts exposed to TGF β 1 alone. In addition, IL1 β decreased the TGF β 1-induced upregulation of lysyl oxidase, an enzyme involved in collagen cross-linking. Furthermore, we found that lung and dermal fibroblasts do not always behave identically towards IL1 β . Suppression of COL1A1 by IL1 β in the presence of TGF β 1 is more pronounced in lung fibroblasts compared to dermal fibroblasts, whereas a higher upregulation of MMP1 is seen in dermal fibroblasts. The role of IL1 β in fibrosis should be reconsidered, and the differences in phenotypical properties of fibroblasts derived from different organs should be taken into account in future anti-fibrotic treatment regimes.

Citation: Mia MM, Boersema M, Bank RA (2014) Interleukin-1 β Attenuates Myofibroblast Formation and Extracellular Matrix Production in Dermal and Lung Fibroblasts Exposed to Transforming Growth Factor- β 1. PLoS ONE 9(3): e91559. doi:10.1371/journal.pone.0091559

Editor: Marie Jose Goumans, Leiden University Medical Center, Netherlands

Received: August 21, 2013; **Accepted:** February 13, 2014; **Published:** March 12, 2014

Copyright: © 2014 Mia et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The authors thankfully acknowledge the financial support of the Netherlands Institute for Regenerative Medicine (NIRM, grant No. FES0908). Microscopical imaging was performed at the UMCG Imaging Center (UMIC), which is supported by the Netherlands Organisation for Health Research and Development (ZonMW grant 40-00506-98-9021). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: R.A.Bank@umcg.nl

Introduction

Fibrosis is the result of defective repair processes often seen after chronic injury and/or inflammation in a large variety of organs and tissues, such as the kidney, heart, liver, lung and skin. IL1 β has been implicated as one of the dominant players in the development of fibrosis [1,2,3,4,5,6,7]. Like various organ fibrosis, lung and skin fibrosis are mediated by the IL1 β [6,7,8]. It is expressed in the acute phase of inflammation, but is also elevated in the later stages of inflammation and tissue repair. The hallmark of fibrosis is an excessive accumulation of extracellular matrix (ECM), especially due to an imbalance between collagen synthesis and degradation [8,9,10,11,12]. One of the key processes in fibrosis is the activation of fibroblasts into myofibroblasts [13], a process that seems to be dependent on the activation of the GLI1 of the Hedgehog pathway [12,14]. The consensus is that myofibroblasts are ultimately responsible for the excessive deposition of ECM in fibrosis.

Various cytokines play a role in the differentiation of fibroblasts into myofibroblasts. One of the major pro-fibrotic cytokines is

transforming growth factor- β (such as TGF β 1), as it induces the differentiation of fibroblasts into myofibroblasts. Myofibroblasts are characterized by the presence of cytoplasmic stress fibers and show an excessive production of collagen [9,15,16,17,18,19,20]. TGF β 1 is also involved in the disbalance regarding the expression of matrix metalloproteinases capable of degrading collagen (e.g. MMP1, -2, -9 and -14) *versus* their inhibitors (e.g. tissue inhibitor of matrix metalloproteinase 1 and -2) [21].

As mentioned, IL1 β influences the severity of fibrosis. Neutralisation or other ways of knocking down of IL1 β *in vivo* results in an attenuation of fibrosis [1,2,3,4,5], whereas increasing the level of IL1 β enhances the severity of fibrosis [2,6,7]. However, the direct effect of IL1 β on fibroblasts is unclear. It has been known for a long time that fibroblasts exposed to IL1 β invariably increase the expression of a variety of MMPs, and subsequently the breakdown of collagen [22,23,24,25,26,27,28]. This anti-fibrotic phenomenon shows a dual role for IL1 β in fibrosis, as this should diminish the excessive accumulation of ECM. Conflicting data exists regarding the direct effect of IL1 β on fibroblasts with respect to collagen expression/synthesis. Elevated levels of collagen expression/

synthesis have been reported [29,30,31], but also opposite data [22,26,27], or no changes at all have been published [32,33,34]. We are not aware of studies regarding the effects of IL1 β on the differentiation of fibroblasts to myofibroblasts, although a number of studies show that IL1 β is able to induce epithelial to mesenchymal transition (EMT) and endothelial to mesenchymal transition (EndoMT), i.e. the formation of myofibroblasts out of epithelial and endothelial cells [10,35,36]. Furthermore, not much is known whether IL1 β and TGF β 1 have co-stimulatory effects, although both cytokines are present during tissue repair processes. Interestingly, about two decades ago it has been reported that IL1 β is able to inhibit collagen type I synthesis in fibroblasts that are exposed to TGF β [32], but this potentially anti-fibrotic finding seems to be overlooked.

In this study we investigated the effects of IL1 β , TGF β 1, and a combination of IL1 β and TGF β on cultured adult human dermal and lung fibroblasts (HDFa and HLFa, respectively). The following questions were addressed: is IL1 β (with or without the presence of TGF β 1) involved in (1) fibroblast to myofibroblast differentiation (including the activation of the Hedgehog pathway), (2) collagen synthesis (including the collagen-modifying enzymes prolyl hydroxylase, lysyl hydroxylase, and lysyl oxidase), and (3) increased levels of MMPs. In addition, we investigated whether fibroblasts derived from different organs (lung, skin) respond equally to pro-fibrotic stimuli. The rationale for this is that fibroblasts from internal organs show dramatic differences in gene expression patterns compared to dermal fibroblasts [37,38].

Materials and Methods

Cell culture

HDFa [CCD-1093Sk (ATCC CRL-2115), ATCC, USA] and HLFa [CCD-19Lu (ATCC CCL-210), ATCC, USA] were cultured in Eagle's minimal essential medium (EMEM) (BE12-662F, Lonza Group Ltd, Switzerland) supplemented with 1% L-glutamine (Lonza, Switzerland), 1% penicillin/streptomycin (Gibco Life Technologies Ltd., UK) and 10% fetal bovine serum (FBS) (Thermo Scientific, USA) at 37°C in 5% CO₂. Passage 7 to 12 of HDFa and HLFa were seeded with a density of 15,000 cells/cm² onto a Costar 12-well plate (for quantitative real time polymerase chain reaction) or a 24-well plate (Corning Inc., USA) (for immunofluorescence staining). Cells were washed with phosphate buffer saline (PBS) after 72 h and starved for 16 h in EMEM supplemented with 1% L-glutamine, 1% penicillin/streptomycin and 0.5% FBS. Cultures were then stimulated with/without recombinant human IL1 β (10 ng/ml) (201-LB-005, R&D Systems Inc, USA), recombinant human TGF β 1 (0.5, 2 and 10 ng/ml) (100–21, Peprotech, UK), or combinations thereof, for the time period as indicated. Subsequently, whole-cell lysates (as obtained with RLT-buffer; Qiagen Inc, USA), cells fixed by 1% para-formaldehyde (PFA; Merck, Germany), or collected conditioned media were used for quantitative real time polymerase chain reaction (qRT-PCR), immunofluorescence staining, and LOX activity tests, respectively.

RNA isolation, cDNA synthesis and qRT-PCR

HDFa and HLFa were treated with IL1 β and/or TGF β 1 for 24 h (or 48 h) or pre-treated with TGF β 1 (48 h) followed by IL1 β stimulation for 48 h. Total RNA was isolated using the RNeasy Kit (Qiagen Inc., USA) according to the manufacturer's protocol. RNA concentration and RNA quality were measured with UV spectrophotometry (NanoDrop Technologies, Wilmington, NC). For the synthesis of cDNA, total RNA was reverse transcribed with the First Strand cDNA synthesis kit (Fermentas UAB, Lithuania)

according to the manufacturer's protocol. Gene expression analysis was performed by means of qRT-PCR in a 10 μ l reaction mixture containing 10 ng cDNA, SYBR Green Supermix (Bio-Rad, USA), 6 μ M forward primer, and 6 μ M reverse primer (for primer sequences see Table 1). qRT-PCR was performed in triplicate for each condition in a 384-well plate at 95°C for 15 sec and 60°C for 1 min for 40 cycles using the ViiA 7 Real-Time PCR System (Applied Biosystems, USA). Data was analysed with the ViiA 7 Real-Time PCR System Software v1.1 (Applied Biosystems, USA).

Immunofluorescence staining for α SMA and SM22 α

HDFa and HLFa were cultured in 24-well plates and treated for 48 h with (1) IL1 β , (2) TGF β 1, or (3) a combination of both. In another experiment, fibroblasts were pre-treated with TGF β 1 (48 h) followed by IL1 β stimulation for 48 h. After treatment, cells were washed with PBS and fixed with 1% PFA in PBS for 15 min at room temperature (RT). Cells were then permeabilized with 0.5% Triton X-100 (Merck, Germany) in PBS for 3 min at RT. Subsequently, cells were washed and incubated for 1 h at RT with (1) monoclonal mouse anti-human α SMA (M0851, Dako, Denmark) or (2) polyclonal rabbit to SM22 α (ab14106, Abcam, UK); both primary antibodies were diluted 1:100 in PBS containing 2% bovine serum albumin (BSA) (K1106, Sanquin reagents, Netherlands). After washing with PBS, cells were incubated for 30 min at RT with biotinylated secondary antibody (1) goat-anti-mouse (1080-08, SouthernBiotech, USA) (1:100) or (2) goat anti-rabbit (E0432, Dako, Denmark) (1:100) diluted in PBS containing 2% BSA for 30 min at RT. The cells were washed again and incubated with streptavidine-CY3 (Invitrogen, USA) (1:100) in PBS containing 1% BSA and DAPI (1:10,000) for 30 min. After washing with PBS, cell culture wells were mounted with citifluor (Agar Scientific, UK) and analyzed by fluorescence imaging microscopy (TissueFAXS, TissueGnostics GmbH, Austria), which is a valid method of quantification of immunofluorescent staining. Briefly, immunofluorescence staining was performed in a 24-well culture plate. The total area of the well was scanned

Table 1. List of primers used for qRT-PCR analysis.

Gene	Forward Sequence	Reverse Sequence
ACTA2	CTGTTCACGACATCCTTCAT	TCATGATGCTGTTGTAGGTGGT
COL1A1	GGGATTCCTCGGACCTAAAG	GGAACACCTCGCTCTCCA
COL3A1	CTGGACCCAGGGTCTTC	CATCTGATCCAGGGTTTCCA
GLI1	CAGGGAGGAAAGCAGACTGA	ACTGCTGCAGGATGACTGG
GLI1ΔN	CCAGACAGAGGCCCACTC	CCCGCTTCTTGTTCAACTT
LOX	GGATACGGCACTGGCTACTT	GACGCTGGATGTAGTAGGG
MMP1	GCTAACCTTTGATGCTATAACTACGA	TTGTGCGCATGTAGAATCTG
MMP2	CCCCAAACGGACAAAGAG	CTTCAGCACAAACAGGTTGC
MMP9	GAACCAATCTCACCGACAGG	GCCACCCGAGTGTAACCATA
MMP14	TACTTCCCAGGCCCAAC	GCCACCAGGAAGATGTCATT
P4HA1	AAGATCTAACAGGACTAGATGTTTCCA	TCCTCCAACTCCATAATTTGC
P4HB	GGAATGGAGACACGGCTTC	TTCAGCCAGTTTCACGATGTC
PLOD1	GAAGCTCTACCCCGGCTACT	CTTGATAGCGGACGACAAAGG
PLOD2	ATGGAAATGGACCCACCAA	TGCAGCCATTATCTGTGTC
TAGLN	GGCCAAGGCTCTACTGTCTG	CCCTTGTTGCCATGTCT
YWHAZ	GCAATTACTGAGAGACAATTGACA	TGGAAGGCCGGTAATTTT

doi:10.1371/journal.pone.0091559.t001

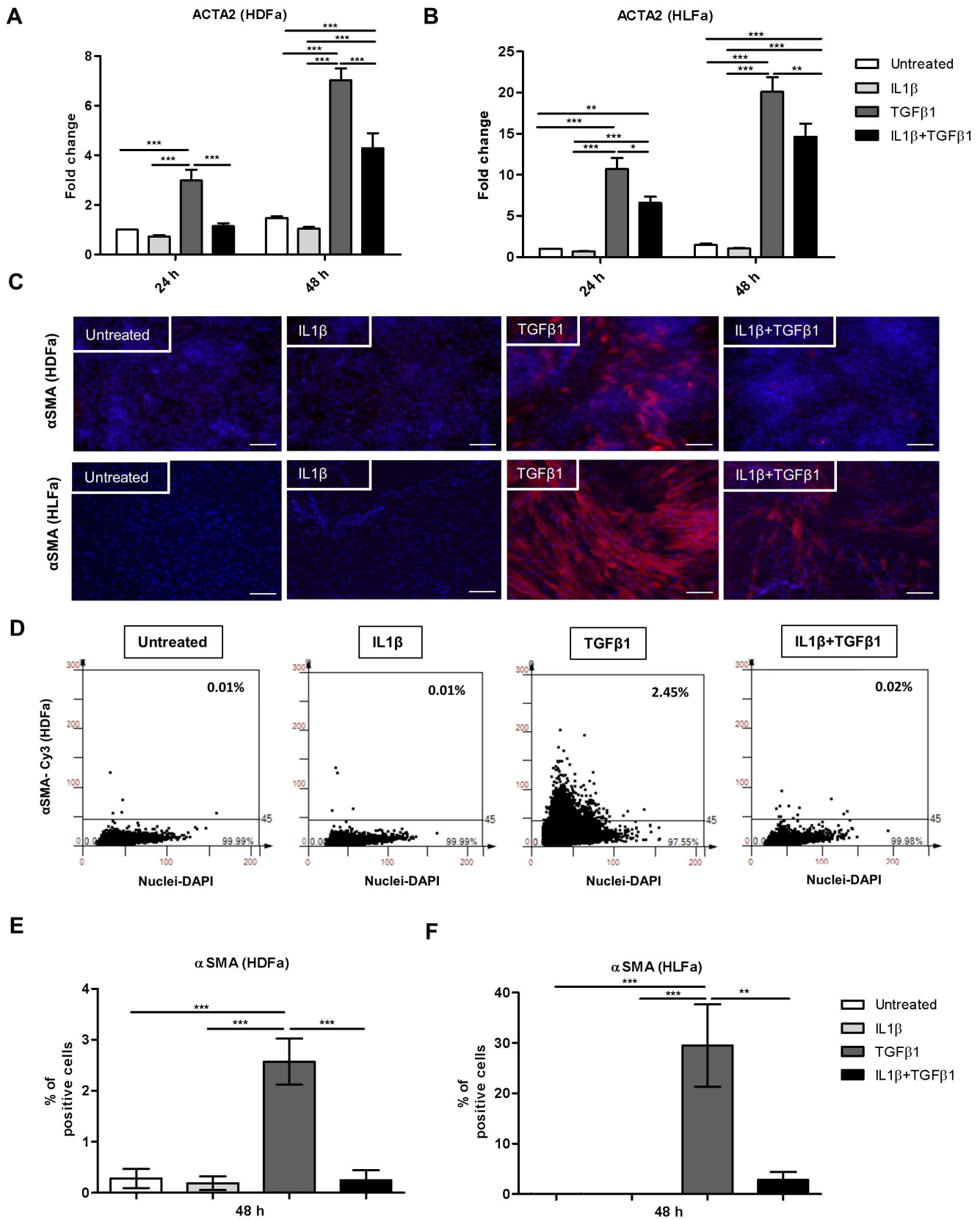


Figure 1. Effects of IL1 β on TGF β 1-induced α SMA synthesis in dermal and lung fibroblasts. HDFa and HLFa were treated with IL1 β , TGF β 1, or a combination of both, for 24 and 48 h. (A–B) The mRNA levels of ACTA2 of HDFa and HLFa were measured with qRT-PCR and expressed as fold change compared to untreated control. (C) Representative immunofluorescence stainings for α SMA are shown for HDFa (upper panel) and HLFa (lower panel). White scale bar represents 100 μ m. (D) Representative TissueFAXS plots showing the % of positive cells (upper right corner) for α SMA positive fibroblasts in HDFa. (E–F) Quantification of the % of cells positive for α SMA are shown for HDFa and HLFa respectively. doi:10.1371/journal.pone.0091559.g001

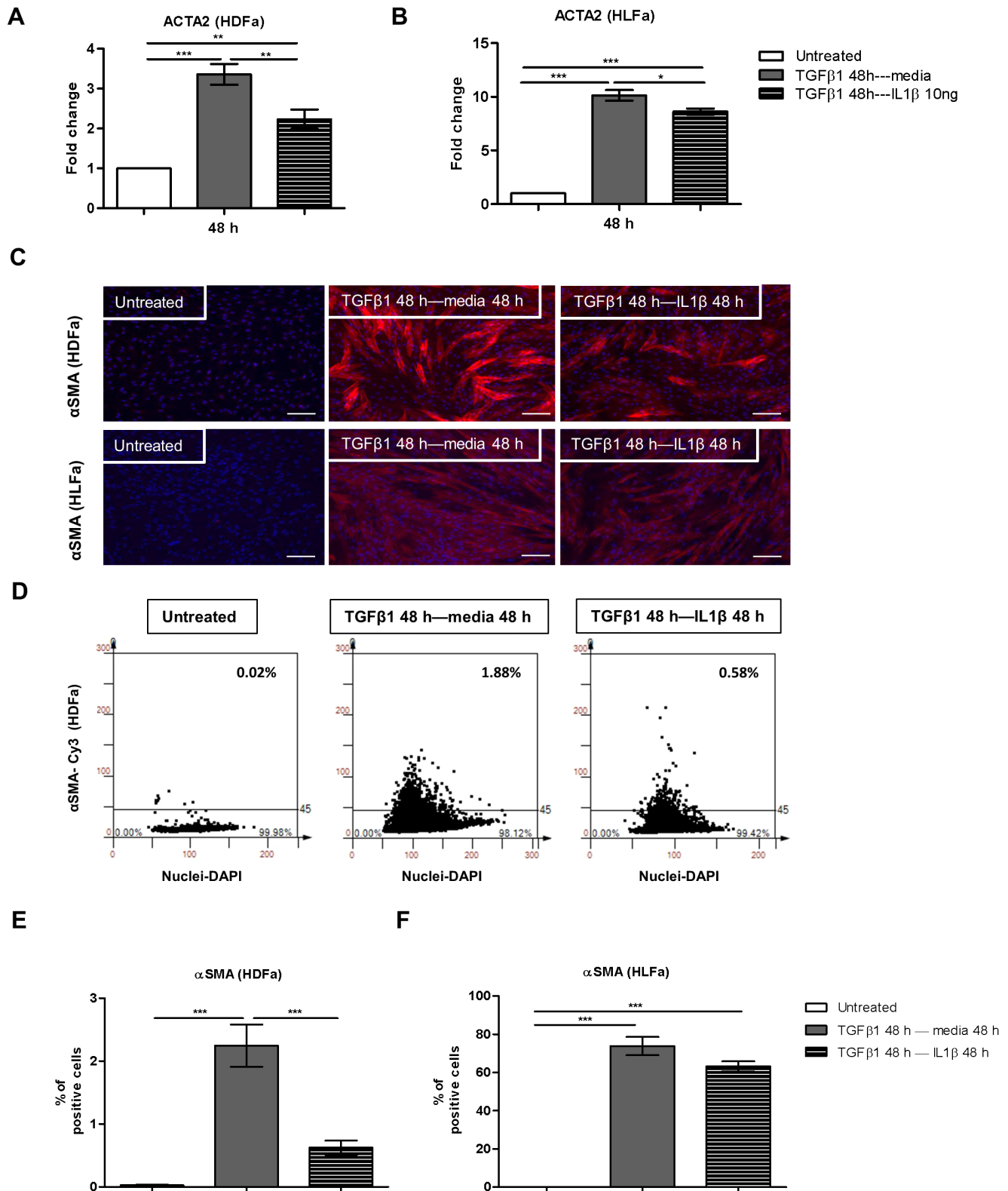


Figure 2. Post-treatment effects of IL1 β on TGF β 1-induced α SMA synthesis in dermal and lung fibroblasts. Fibroblasts were pre-treated with TGF β 1 for 48 h followed by IL1 β for 48 h. (A–B) The mRNA levels of ACTA2 of HDFa and HLFa were measured with qRT-PCR and expressed as fold change compared to untreated control. (C) Representative immunofluorescence stainings for α SMA are shown for HDFa (upper panel) and HLFa (lower panel). White scale bar represents 100 μ m. (D) Representative TissueFAXS plots showing the % of positive cells (upper right corner) for α SMA positive fibroblasts in HDFa. (E–F) Quantification of the % of cells positive for α SMA are shown for HDFa and HLFa respectively. doi:10.1371/journal.pone.0091559.g002

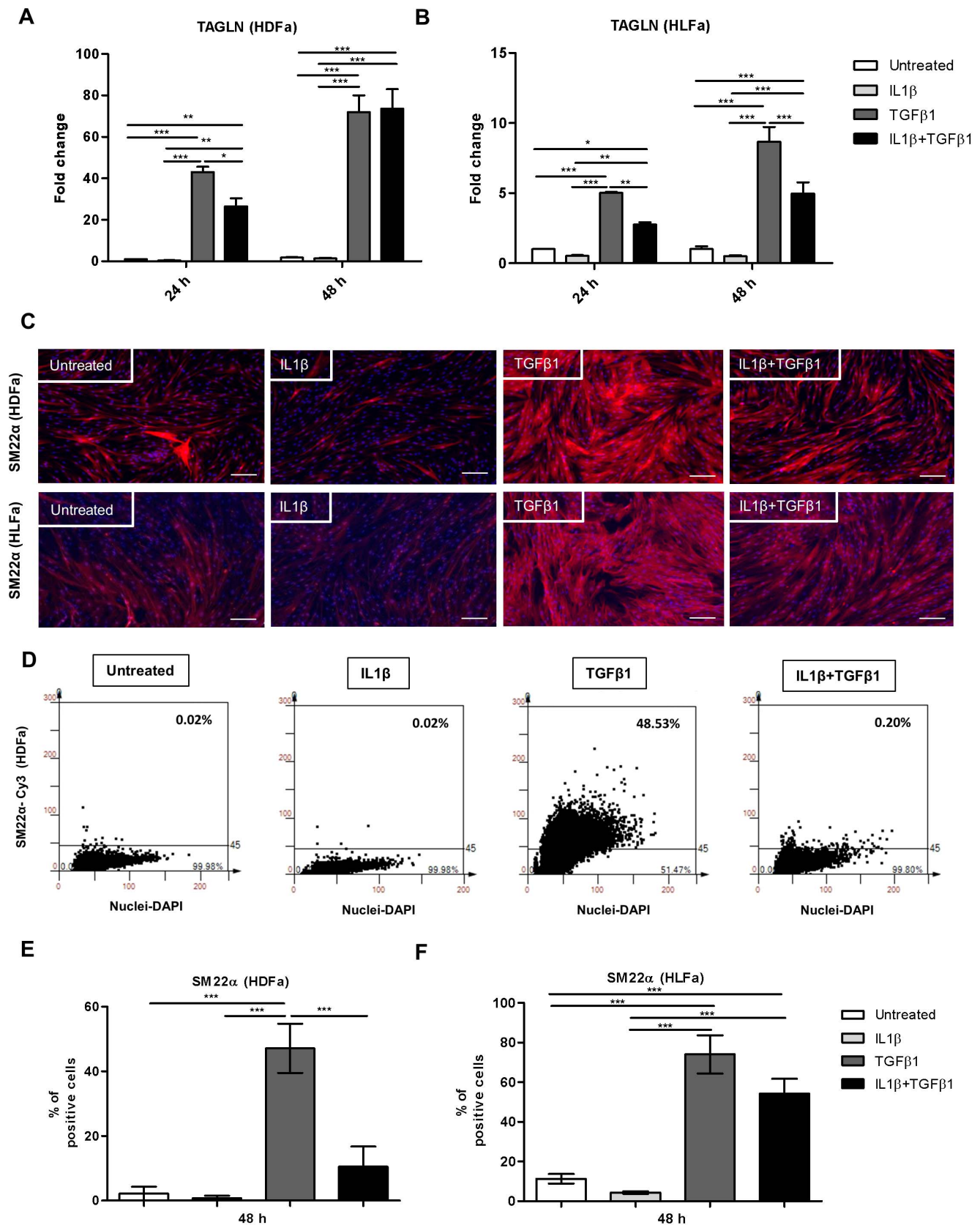


Figure 3. Effects of IL1 β on TGF β 1-induced SM22 α synthesis in dermal and lung fibroblasts. HDFa and HLFa were treated with IL1 β , TGF β 1, or a combination of both, for 24 and 48 h. (A–B) The mRNA levels of TAGLN of HDFa and HLFa were measured with qRT-PCR and expressed as fold change compared to untreated control. (C) Representative immunofluorescence stainings for SM22 α are shown for HDFa (upper panel) and HLFa (lower panel). White scale bar represents 100 μ m. (D) Representative TissueFAXS plots showing the % of positive cells (upper right corner) for SM22 α positive fibroblasts in HDFa. (E–F) Quantification of the % of cells positive for SM22 α are shown for HDFa and HLFa respectively. doi:10.1371/journal.pone.0091559.g003

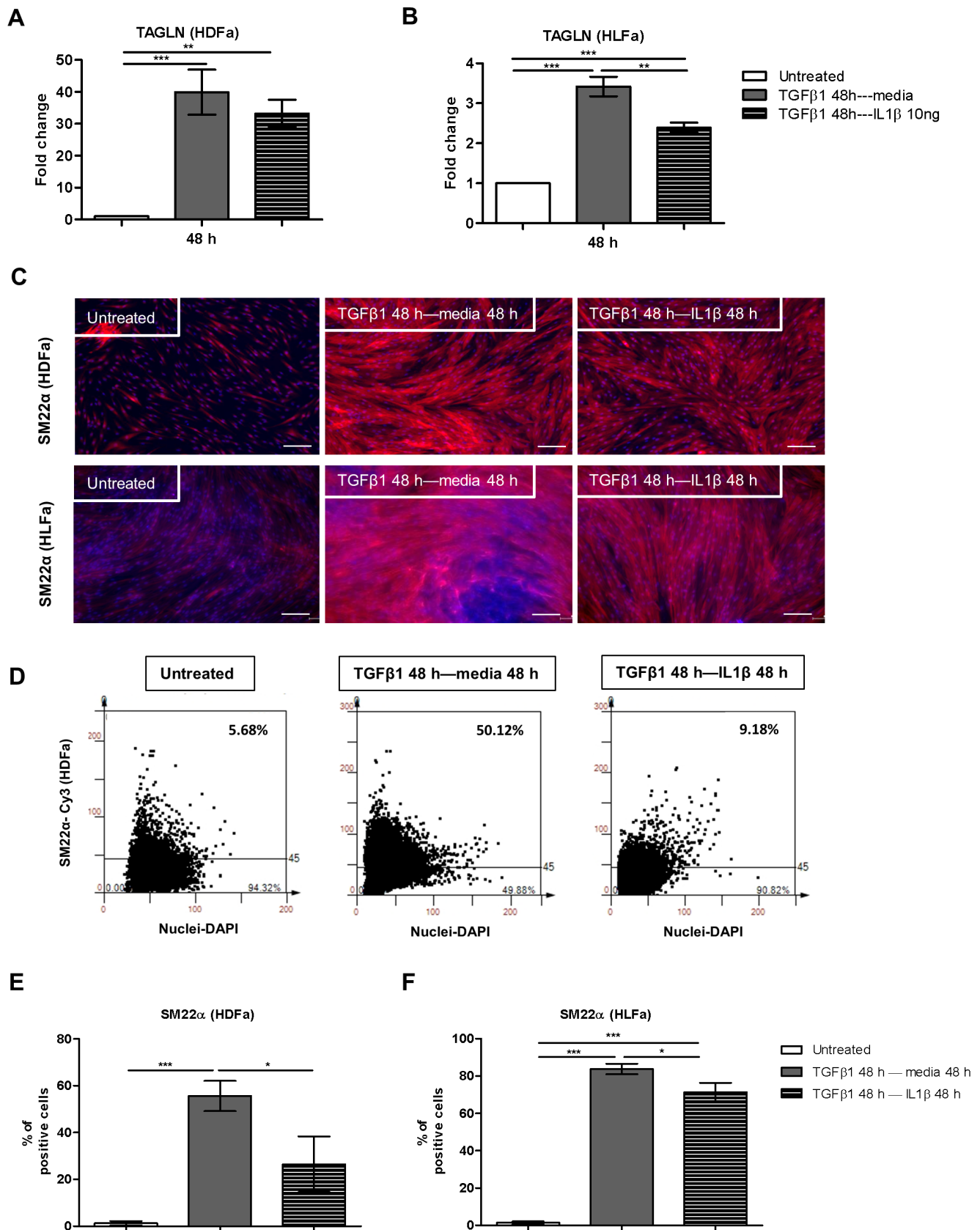


Figure 4. Post-treatment effects of IL1 β on TGF β 1-induced SM22 α synthesis in dermal and lung fibroblasts. Fibroblasts were pre-treated with TGF β 1 for 48 h followed by IL1 β for 48 h. (A–B) The mRNA levels of TAGLN of HDFa and HLFa were measured with qRT-PCR and expressed as fold change compared to untreated control. (C) Representative immunofluorescence stainings for SM22 α are shown for HDFa (upper

panel) and HLFa (lower panel). White scale bar represents 100 μ m. (D) Representative TissueFAXS plots showing the % of positive cells (upper right corner) for SM22 α positive fibroblasts in HDFa. (E–F) Quantification of the % of cells positive for SM22 α are shown for HDFa and HLFa respectively. doi:10.1371/journal.pone.0091559.g004

by the TissueFAXS after which we used the TissueQuest software to quantify the total number of nuclei and the number of nuclei positive for the protein visualized in that particular experiment. The TissueQuest quantification results in scattergram. Each dot in a scattergram represent a nucleus and the fluorescent intensity as a measure for the amount of protein present of that particular cell can be determined on the Y-axis. Data are presented as a percentage of positive cells (mean \pm SEM) of four to six independent experiments.

Collagen type I staining

HDFa and HLFa were treated for 7 days with (1) IL1 β , (2) TGF β 1, or (3) both. Additionally, Vitamin C (Sigma-Aldrich, USA) was added to the cell culture media. For collagen type I detection, we followed the same protocol as described above to detect α SMA and SM22 α . To observe the production of intracellular and extracellular collagen, stainings were performed with or without Triton x-100 (Merck, Germany) permeabilization. Monoclonal mouse to collagen type I (1:100) (ab90395, Abcam, UK) was used as primary antibody, and a biotinylated goat-anti-mouse (1:100) as secondary antibody (SouthernBiotech, USA). Collagen type I immunofluorescence was visualized using TissueFAXS as described above.

LOX activity assay

To measure the activity of LOX, conditioned media of fibroblasts treated for 24 and 48 h with (1) IL1 β , (2) TGF β 1, or (3) both were used. LOX activity was determined with the Amplitude Fluorimetric Lysyl Oxidase Assay Kit (AAT Bioquest Inc, USA) in accordance to the manufacturer's protocol. Briefly, 50 μ l of assay reaction mixture was prepared, followed by the addition of 50 μ l of conditioned media. The mixture was incubated at 37°C for 20 min; fluorescence intensity was detected at Excitation/Emission 540/590 nm with a Varioskan Flash Multimode Reader. Background fluorescence was subtracted and the conditioned media values were calculated and expressed as relative fluorescence intensity (RFU). To present the LOX activity, value of each treatment were plotted as bar graph. Data are presented as mean \pm SEM for two independent experiments. The whole reaction was performed in a black 96-well plate.

Statistics

All mRNA data were normalized against the reference gene tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ). Data are presented as mean \pm SEM for at least three independent experiments. All immunofluorescence data are presented as mean \pm SEM for at least four independent experiments. Results were analysed either with a one-way analysis of variance (ANOVA) followed by Tukey's post-test analysis or two-way ANOVA followed by Bonferroni post-test analysis using Graph-Pad Prism Version 5 (GraphPad Software Inc., USA). $P < 0.05$ was considered to be statistically significant. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Results

Dose-dependent effect of cytokines

To investigate the optimal concentration of TGF β 1, we performed a dose-dependent titration experiment with three concentrations of TGF β 1 (0.5, 2 and 10 ng/ml) in combination

with 10 ng/ml of IL1 β for 48 hours on both dermal and lung fibroblasts. Gene expression analysis showed that stimulation of fibroblasts with 0.5 ng of TGF β 1 did not increase the expression of ACTA2 in HDFa (Fig. S1A). However, 0.5 ng of TGF β 1 was sufficient to significantly increase the expression of ACTA2 in HLFa (Fig. S1B). A higher dose of TGF β 1 (2 ng/ml) did increase the expression of ACTA2 in both types of fibroblasts (Fig. S1A–B), as well as that of other genes (GLI1, COL1A1, PLOD2) that are associated with the differentiation of fibroblasts into myofibroblasts (Fig. S1C–F and S2A–B). However, both in HDFa and HLFa, the increase in gene expression levels as a result of stimulation with 2 ng/ml of TGF β 1 often did not reach the levels of the experiments with 10 ng/ml TGF β 1 (for example Fig. 1A–B).

In the titration experiment, IL1 β did not reduce the expression of ACTA2 in HLFa (Fig. S1B) and GLI1 in HDFa (Fig. S1C) when fibroblasts were treated with either 0.5 or 2 ng/ml of TGF β 1, although this was seen for 10 ng/ml (for example Fig. 1A–B). However, IL1 β was able to downregulate the TGF β 1-induced COL1A1 gene expression on both types of fibroblast (Fig. S1E–F). We did find a dose-dependent effect of TGF β 1 on the gene expression levels of MMP1 (Fig. S2C–D).

These findings suggest that a lower dose of TGF β 1 was sufficient to increase the gene expression of several genes associated with myofibroblast differentiation and matrix remodelling. However, the increase in gene expression after stimulation with 0.5 and 2 ng/ml of TGF β 1 was quite limited for some genes. Therefore, throughout this paper, we describe the findings of the effects of 10 ng/ml TGF β 1 and IL1 β .

IL1 β inhibits TGF β 1-induced α SMA synthesis

TGF β 1 is the principal cytokine involved in the transition of fibroblasts into myofibroblasts [15,39]. Myofibroblasts show, in contrast to fibroblasts, extensive cytoplasmic stress fibers with alpha smooth muscle actin (α SMA) and smooth muscle protein 22-alpha (SM22 α) as its components [40,41,42]. In order to examine the effect of IL1 β on TGF β 1-induced α SMA expression, fibroblasts were treated either with IL1 β , TGF β 1, or both for 24 or 48 h (Fig. 1A–F). In addition, cells were pre-treated with TGF β 1 for 48 h followed by incubation with IL1 β for 48 h (Fig. 2A–F). The results showed that IL1 β alone did not have an effect on smooth muscle actin alpha 2 (ACTA2) mRNA expression compared to untreated cells (Fig. 1A–B). However, ACTA2 expression was significantly increased in TGF β 1-stimulated cells over time, and ACTA2 gene expression was significantly decreased when the two cytokines were combined in HDFa and HLFa at both time points (Fig. 1A–B). Even post-treatment with IL1 β significantly decreased the TGF β 1-induced ACTA2 gene expression (Fig. 2A–B). To confirm the mRNA expression data on protein level, changes in cytoskeleton structure of fibroblasts and myofibroblasts were investigated by immunofluorescence staining for α SMA. In line with the mRNA data, IL1 β alone had no effect on actin stress fiber formation in quiescent HDFa and HLFa, and stimulation with TGF β 1 resulted in the formation of actin stress fibers in both cell types (Fig. 1C–F). Furthermore, in a TGF β 1-rich environment, IL1 β counteracted the TGF β 1-induced actin stress fiber formation, both in a combined (Fig. 1C–F) and in a post-treatment fashion in both HLFa and HDFa (Fig. 2C–F). Thus, from the α SMA data we conclude that IL1 β is able to diminish TGF β 1-induced myofibroblast formation.

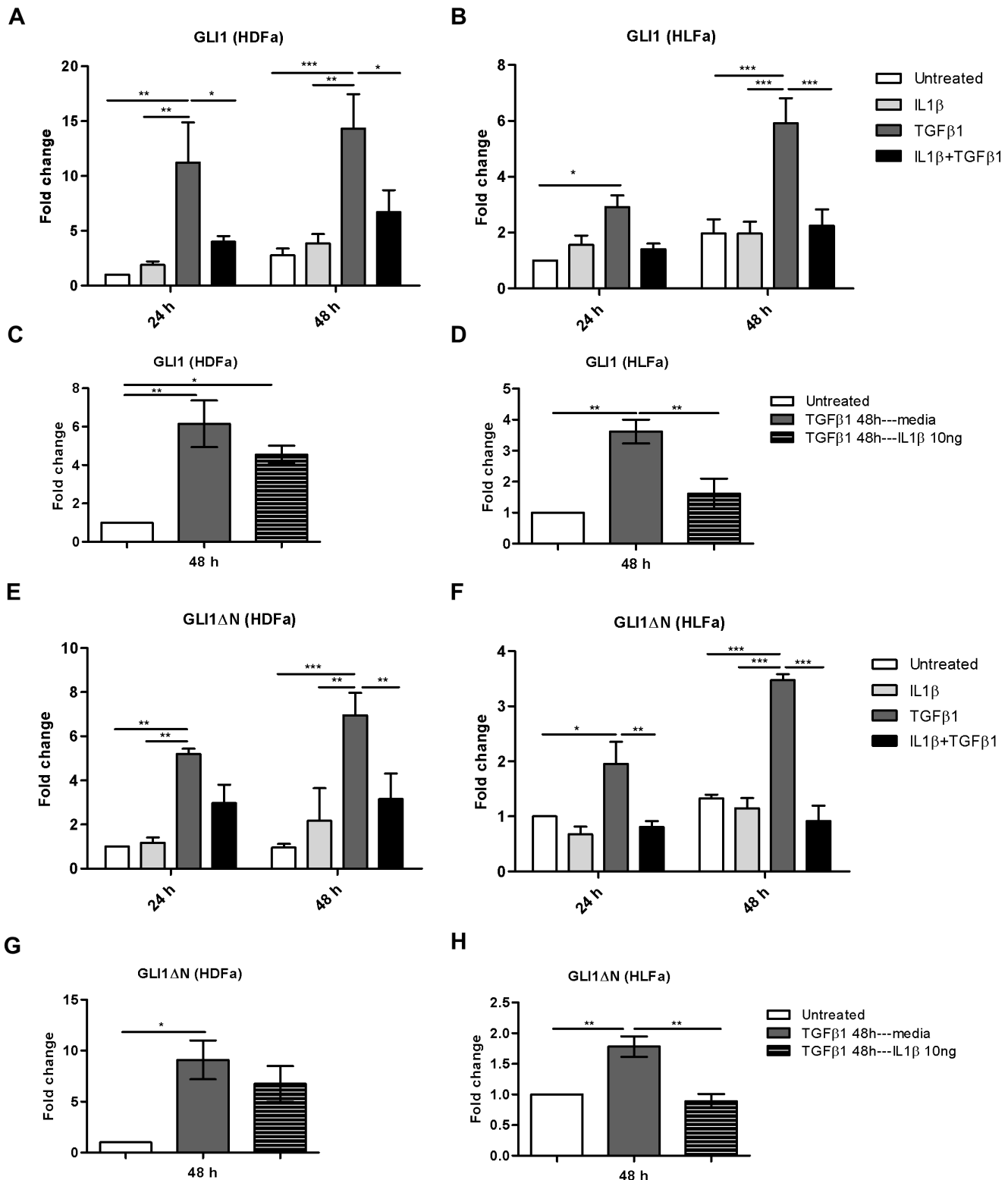


Figure 5. Effects of IL1 β on TGF β 1-induced gene expression of Hedgehog pathway effector GLI1 and its isoform GLI1 Δ N in dermal and lung fibroblasts. (A–B) HDFa and HLFa were treated with IL1 β , TGF β , or a combination of both for 24 and 48 h. The mRNA levels of GLI1 were measured with qRT-PCR and expressed as fold change compared to untreated control. (C–D) The mRNA level of GLI1 in HDFa and HLFa. Fibroblasts were pre-treated with TGF β 1 for 48 h followed by IL1 β for 48 h and quantified by qRT-PCR and expressed as fold change compared to untreated control. (E–F) HDFa and HLFa were treated with IL1 β , TGF β 1, or a combination of both, for 24 and 48 h. The mRNA levels of GLI1 Δ N were measured with qRT-PCR and expressed as fold change compared to untreated control. (G–H) The mRNA level of GLI1 Δ N in HDFa and HLFa. Fibroblasts were pre-treated with TGF β 1 for 48 h followed by IL1 β for 48 h and quantified by qRT-PCR and expressed as fold change compared to untreated control. doi:10.1371/journal.pone.0091559.g005

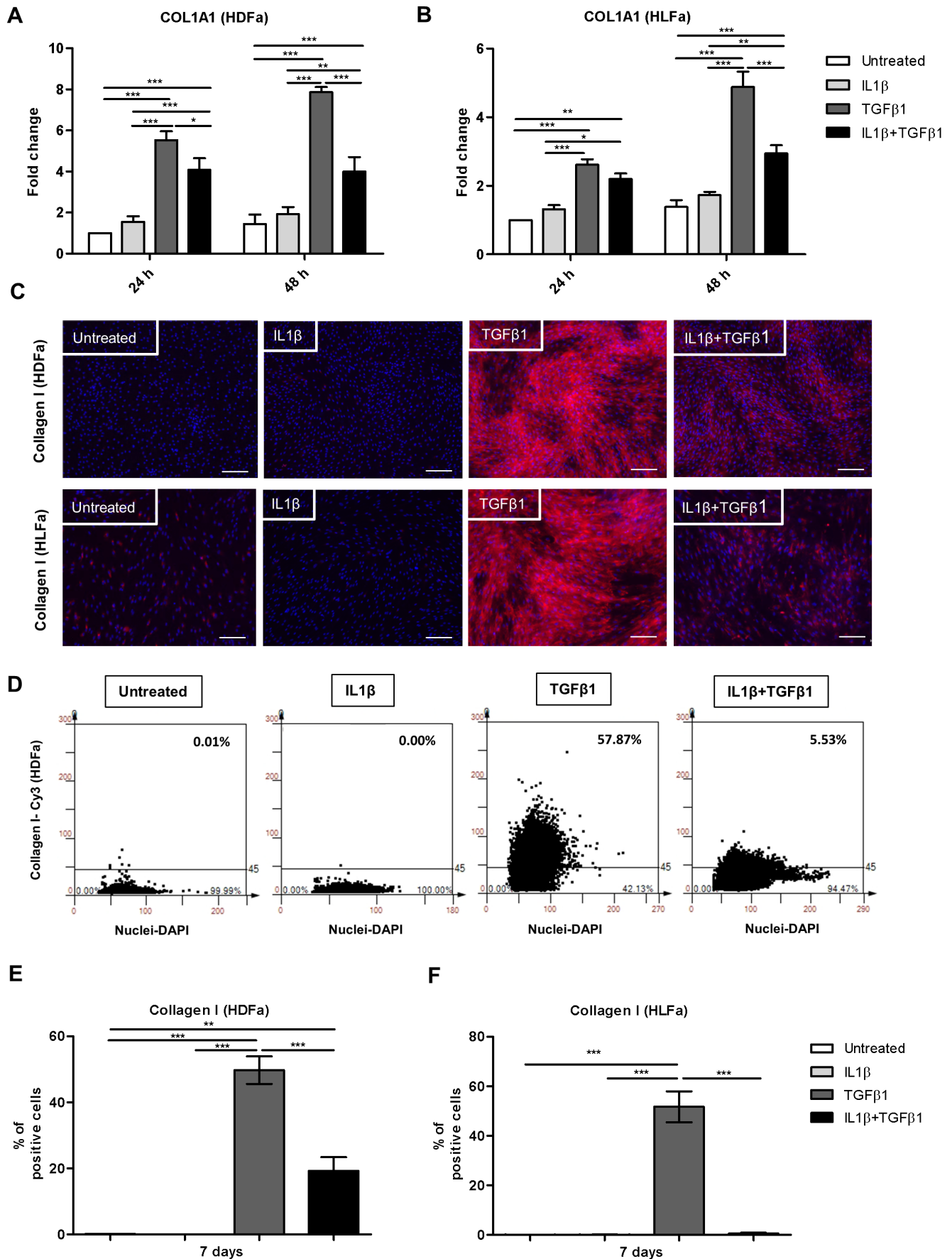


Figure 6. Effects of IL1 β on TGF β 1-induced collagen type I synthesis in dermal and lung fibroblasts. (A–B) HDFa and HLFa were treated with IL1 β , TGF β 1, or a combination of both, for 24 and 48 h. The mRNA levels of COL1A1 were measured with qRT-PCR and expressed as fold change compared to untreated control. (C) Visualisation of synthesized collagen (intracellular) by HDFa (upper panel) and HLFa (lower panel) treated for 7 days with IL1 β , TGF β 1 and in combination thereof. White scale bar of each image represents 100 μ m. (D) Representative TissueFAXS plots showing the % of positive cells (upper right corner) for intracellular collagen type I in HDFa. (E–F) Quantification of the % of cells positive for intracellular collagen type I are shown for HDFa and HLFa respectively. doi:10.1371/journal.pone.0091559.g006

IL1 β inhibits TGF β 1-induced SM22 α synthesis

Although α SMA is the most prominent marker of myofibroblasts, these cells are also characterized by elevated levels of SM22 α , which is an actin isoform. We therefore evaluated whether IL1 β has similar effects on SM22 α . Fibroblasts treated with IL1 β already showed a trend of decreasing tagln (TAGLN) mRNA levels in quiescent HLFa (Fig. 3B). As expected, an upregulation of TAGLN was seen in TGF β 1-treated cells. As observed with ACTA2, treatment with a combination of IL1 β and TGF β 1 caused a significant downregulation of TAGLN gene expression in HDFa after 24 h and in HLFa after 24 h or 48 h (Fig. 3A–B), although no downregulation was seen for HDFa at 48 h. Immunofluorescence studies showed that untreated fibroblasts are SM22 α positive, however upon IL1 β stimulation the intensity of SM22 α staining decreased (Fig. 3C–F). TGF β 1 stimulation highly increased the SM22 α staining in HDFa and HLFa, which was significantly inhibited in HDFa in the presence of IL1 β (Fig. 3C–F). Post-treatment with IL1 β for 48 h significantly reduced the TGF β 1-induced upregulation of TAGLN mRNA expression in HLFa (Fig. 4B), but not in HDFa (Fig. 4A). The protein analysis revealed that post-treatment with IL1 β reversed the TGF β 1-induced SM22 α protein levels both in HDFa and HLFa (Fig. 4C–F). Thus, also the SM22 α data shows that IL1 β is able to diminish the TGF β 1-induced myofibroblast formation.

IL1 β reduces TGF β 1-induced GLI1 and GLI1 Δ N gene expression

Given the fact that GLI1 is required for the differentiation of fibroblasts into myofibroblasts [12,14,43], we tested whether the attenuation by IL1 β of TGF β 1-induced activation of fibroblasts is indeed paralleled by decreased levels of GLI1. HDFa and HLFa were exposed to IL1 β , TGF β 1, or both, for 24 h and 48 h (Fig. 5A–B). No differences were detected in GLI1 mRNA levels between IL1 β -stimulated and untreated fibroblasts at both time points. GLI1 mRNA levels were significantly increased (\sim 10-fold in HDFa and \sim 3-fold in HLFa) after TGF β 1 treatment (Fig. 5A–B). Interestingly, incubation with a combination of IL1 β and TGF β 1 significantly reduced the mRNA expression of GLI1 in HDFa compared to TGF β 1 alone (Fig. 5A), whereas IL1 β only significantly suppressed GLI1 expression at 48 h in HLFa (Fig. 5B), although a trend was seen at 24 h. HLFa treated with TGF β 1 for 48 followed by IL1 β stimulation for 48 h showed a significant reduction in expression of GLI1; while only a trend was seen for HDFa (Fig. 5C–D). Thus, inhibition of myofibroblast differentiation by IL1 β is partially reflected in decreased GLI1 levels.

Recently, an isoform of GLI1, named GLI1 Δ N, has been identified [44]. However, there is limited evidence about its expression and function in fibroblasts and myofibroblasts. We asked whether GLI1 Δ N also respond to IL1 β - and TGF β 1-treated fibroblasts. As was the case with GLI1, the GLI1 Δ N mRNA level was not affected after IL1 β treatment, and was significantly upregulated up to \sim 6-fold in HDFa and up to \sim 3-fold in HLFa after TGF β 1 stimulation (Fig. 5E–F). Treatment of fibroblasts with IL1 β and TGF β 1 together significantly decreased the expression of GLI1 Δ N at 48 h compared to TGF β 1 alone (Fig. 5E–F). This

was also observed when fibroblasts were treated first with TGF β 1 for 48 h, and treated afterwards for 48 h with IL1 β (Fig. 5G–H), although a significance was only reached for HLFa. These findings suggest that in a pro-fibrotic environment the response of GLI1 Δ N to cytokine IL1 β is similar to that of GLI1.

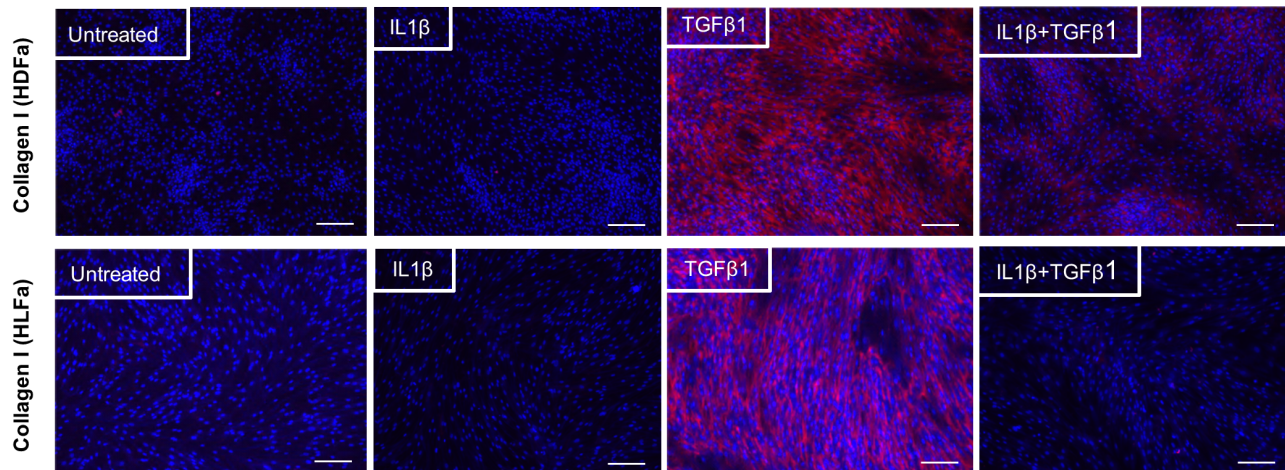
IL1 β decreases TGF β 1-induced collagen type I synthesis, while it increases the expression of COL3A1

Under fibrogenic conditions, myofibroblasts are responsible for the production of excessive ECM. TGF β 1 has been reported to induce accumulation of collagen type I [19,45,46,47]. As we have shown above, IL1 β reversed the TGF β 1-induced α SMA and SM22 α expression. However, the presence of α SMA or SM22 α is not required for myofibroblasts to deposit ECM. We therefore investigated whether IL1 β is also able to reduce the production of ECM molecules, such as collagen, in a TGF β -rich pro-fibrotic environment. Compared to untreated fibroblasts, IL1 β did not alter mRNA levels of collagen type I alpha 1 (COL1A1), whereas TGF β 1 upregulated COL1A1 up to \sim 5- to 7-fold in HDFa (Fig. 6A) and \sim 2- to 4-fold in HLFa (Fig. 6B) at 24 h to 48 h, respectively. A considerable reduction of COL1A1 expression was detected at 48 h when cells were treated with a combination of TGF β 1 and IL1 β (Fig. 6A–B). As expected from the mRNA data, a collagen type I staining by means of immunofluorescence revealed a strong expression both intracellular (Fig. 6C–F) and extracellular (Fig. 7A–D) in either type of fibroblasts treated with TGF β 1 alone. A considerable reduction of collagen type I staining was seen when cells were co-stimulated with IL1 β (Fig. 6C and 7A). Quantification of the % of cells stained positive for intracellular collagen after 7 days demonstrated that IL1 β alone had no effect, that TGF β 1 notably induced collagen synthesis, and that the combination of TGF β 1 with IL1 β showed a clear trend of decreasing collagen type I synthesis in both HDFa and HLFa (Fig. 6D–F). On the other hand, quantification of the % of cells positive for extracellular deposited, collagen type I after 7 days revealed that IL1 β almost completely abolished the TGF β 1-induced collagen type I production in HLFa, whereas a trend was seen for HDFa (Fig. 7A–D).

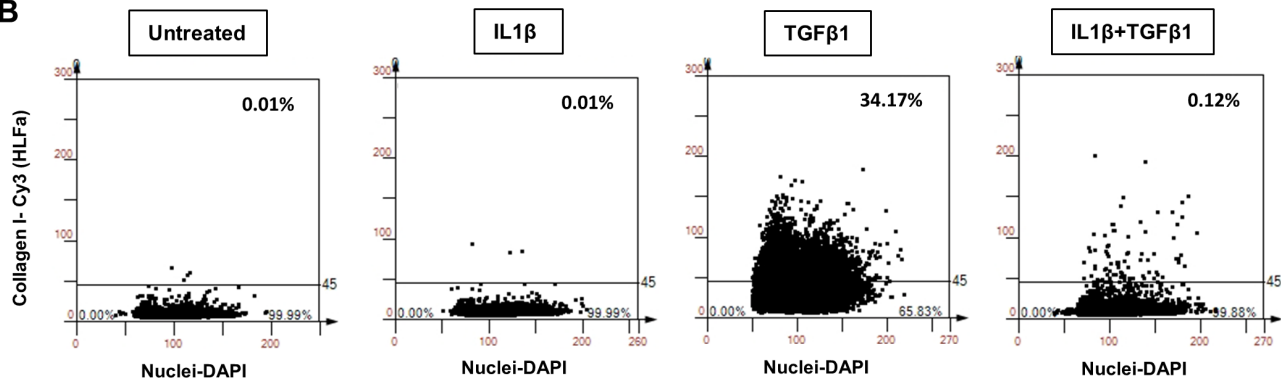
Post-treatment with IL1 β also significantly decreased the mRNA level of COL1A1 in HLFa pre-treated with TGF β 1 (Fig. 7F). However, under the same conditions, no such decrease in COL1A1 mRNA levels was seen in HDFa (Fig. 7E).

Interestingly, an opposite effect was observed for collagen type III alpha 1 (COL3A1) gene expression. Either IL1 β or TGF β 1-treatment significantly increased the mRNA level of COL3A1 in HDFa at both time points (at 24 and 48 h) (Fig. 8A) and in HLFa at 48 h of incubation (Fig. 8B). The fold-increase in COL3A1 mRNA levels compared to untreated cells was higher in HDFa than in HLFa. A combination of the cytokines resulted mRNA levels of COL3A1 that were the same (in HLFa) or even higher (in HDFa) compared to fibroblasts that were treated with TGF β 1 alone (Fig. 8A–B). This was also observed when fibroblasts were treated first with TGF β 1 for 48 h, and treated afterwards for 48 h with IL1 β (Fig. 8C–D). Thus, co-treatment or post-treatment with IL1 β never resulted in a downregulation in mRNA expression of COL3A1, which is in contrast to what was seen with COL1A1.

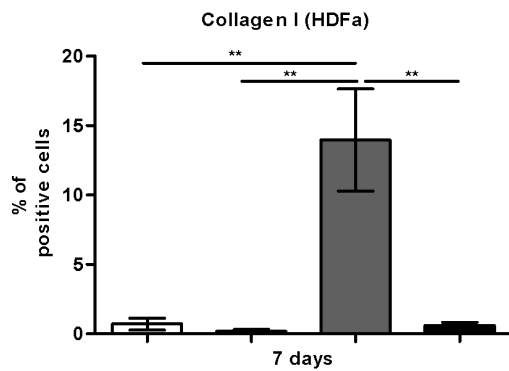
A



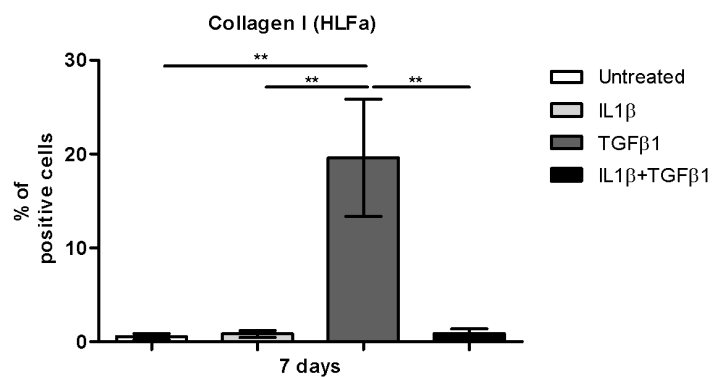
B



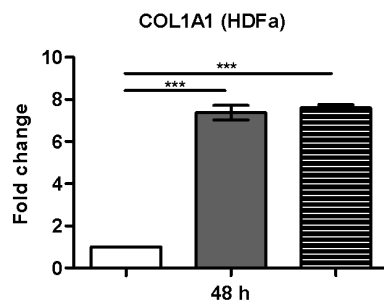
C



D



E



F

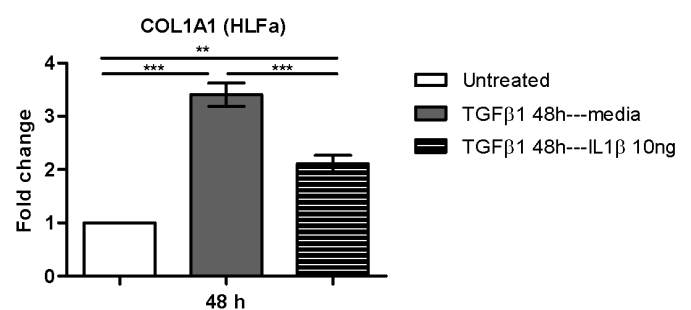


Figure 7. Effects of IL1 β on TGF β 1-induced production of extracellular collagen type I in dermal and lung fibroblasts. (A) Visualisation of deposited extracellular collagen by HDFa (upper panel) and HLFa (lower panel) treated for 7 days with IL1 β , TGF β 1 and in combination thereof. White scale bar of each image represents 100 μ m. (B) Representative TissueFAXS plots showing the % of positive cells (upper right corner) for extracellular collagen type I in HDFa. (C–D) Quantification of the % of cells surrounded by extracellular collagen type I protein are shown for HDFa and HLFa respectively. (E–F) Fibroblasts were pre-treated with TGF β 1 for 48 h followed by IL1 β for 48 h. The mRNA levels of COL1A1 of HDFa and HLFa were measured with qRT-PCR and expressed as fold change compared to untreated control.

doi:10.1371/journal.pone.0091559.g007

IL1 β and TGF β 1 ameliorate P4HA1, P4HB, PLOD1 and PLOD2 gene expression

In the previous paragraph we showed that IL1 β by itself does not affect COL1A1 expression, whereas TGF β 1 increases COL1A1 expression and that IL1 β was able to attenuate the TGF β 1-induced expression of COL1A1. It should be noted that processing of collagen type I (as well as the other collagen types) is a complex process as several enzymes are involved in the processing and modification of collagen. Some of these enzymes play a key role in the stability of the collagen molecule/fibril. In fibrosis, an increase in the expression of collagen-modifying enzymes such as prolyl hydroxylase (e.g. P4H1, P4HB) and lysyl hydroxylase (e.g. PLOD1 and -2) has been shown in e.g. lung and skin fibrosis [48,49,50,51]. TGF β 1 is able to stimulate the expression of the mentioned enzymes [52]. However, the effect of IL1 β on the gene expression level of P4HA, P4HB, PLOD1 and PLOD2 is unknown. We investigated whether IL1 β is able to counteract the TGF β 1-induced overexpression of these enzymes, as we have observed for COL1A1. Gene expression analysis

(Fig. 9A–H) demonstrate that IL1 β , like TGF β 1, significantly upregulates the expression of these enzymes in quiescent fibroblasts both at 48 h, and often also at 24 h (Fig. 9A–H). Treatment with a combination of IL1 β and TGF β 1 did not result in a decrease in expression of the enzymes (which is in contrast with COL1A1); in fact, mRNA levels remained the same or were even higher compared to fibroblasts that were treated with TGF β 1 alone. The most notable increase in expression was observed of PLOD2: the expression of this enzyme was synergistically induced by the combination of IL1 β with TGF β 1 (Fig. 9G–H). This was also observed when fibroblasts were treated first with TGF β 1 for 48 h, and treated afterwards for 48 h with IL1 β (Fig. 9I–J). The enzyme telopeptide lysyl hydroxylase, which is encoded by PLOD2, specifically hydroxylates the lysine residues in the telopeptides, giving rise to the formation of hydroxyallysine-derived cross-links (such as pyridinoline) at the expense of allysine-derived cross-links. Pyridinoline cross-links are increased in fibrosis and seem to be associated with its irreversibility [53,54].

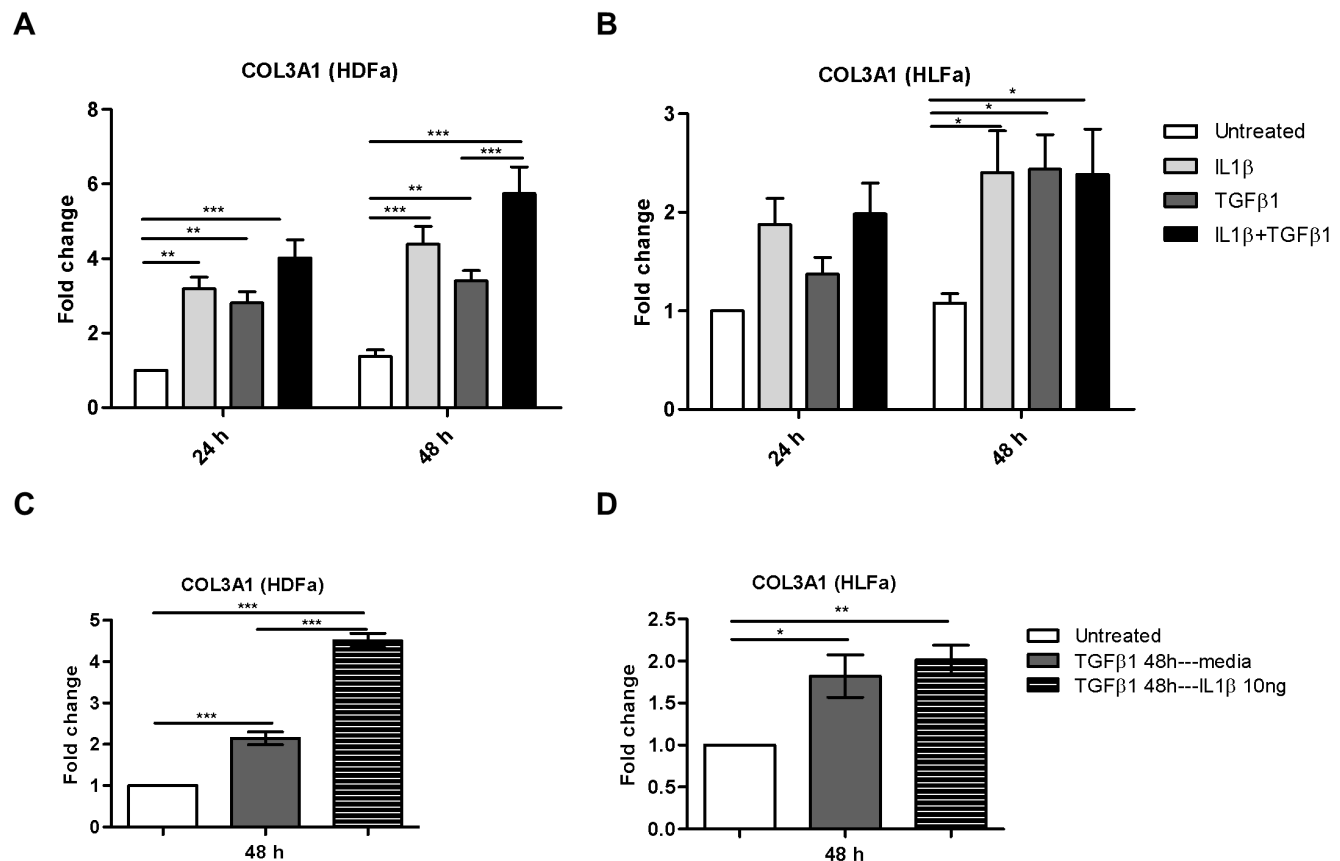


Figure 8. Effects of IL1 β and TGF β 1 on the expression of collagen type III in dermal and lung fibroblasts. (A–B) HDFa and HLFa were treated with IL1 β , TGF β 1, or a combination of both, for 24 and 48 h. The mRNA levels of COL3A1 were measured with qRT-PCR and expressed as fold change compared to untreated control. (C–D) The mRNA level of COL3A1 in HDFa and HLFa. Fibroblasts were pre-treated with TGF β 1 for 48 h followed by IL1 β for 48 h and quantified by qRT-PCR and expressed as fold change compared to untreated control.

doi:10.1371/journal.pone.0091559.g008

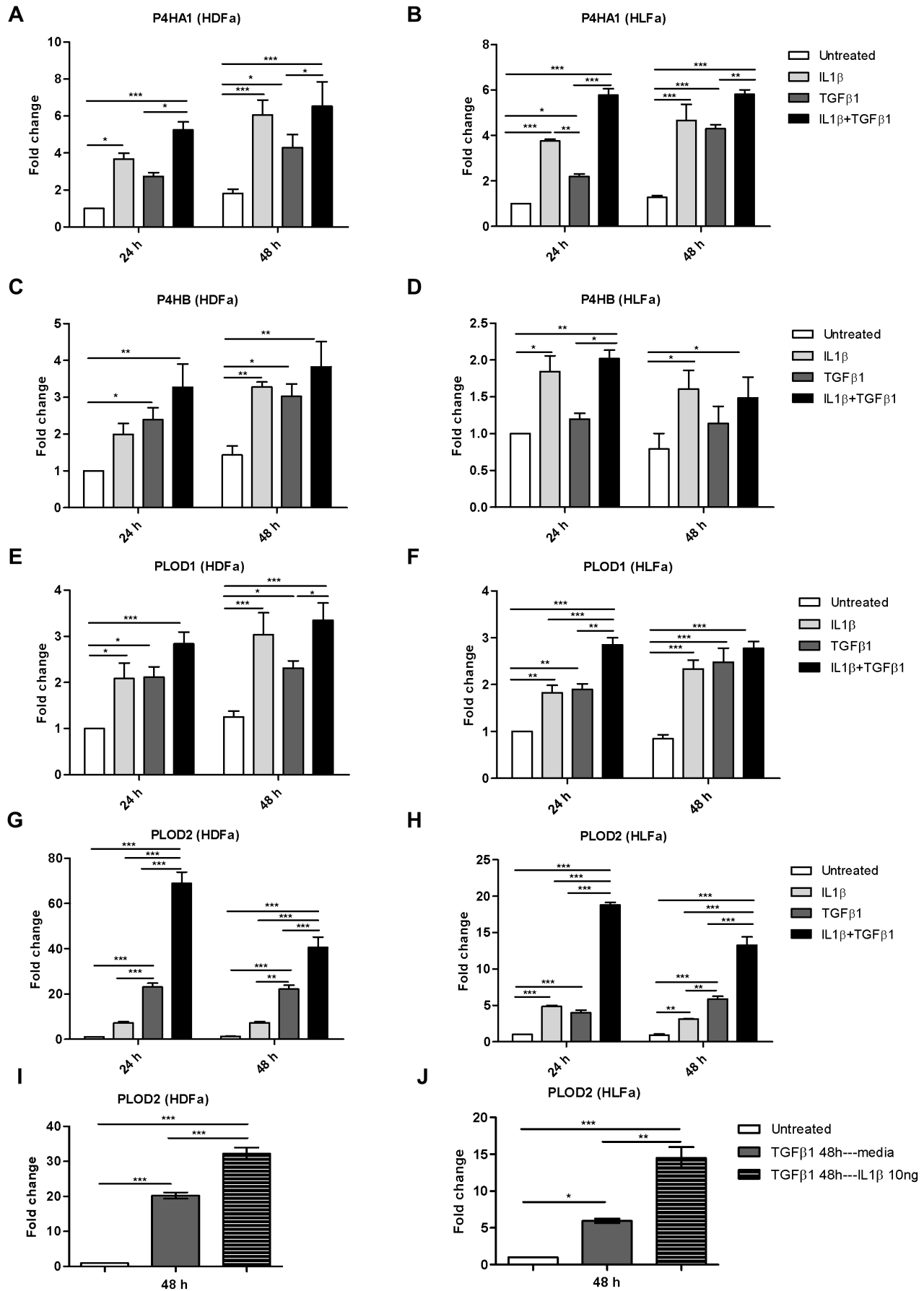


Figure 9. Effects of IL1 β and TGF β 1 on the expression of the intracellular collagen-modifying enzymes prolyl hydroxylase (P4HA1, P4HB) and lysyl hydroxylase (PLOD1, PLOD2) in dermal and lung fibroblasts. (A–H) HDFa and HLFa were treated with IL1 β , TGF β 1, or a combination of both, for 24 and 48 h. The mRNA levels of P4HA1, P4HB, PLOD1 and PLOD2 were measured with qRT-PCR and expressed as fold change compared to untreated control. (I–J) The mRNA levels of PLOD2 in HDFa and HLFa. Fibroblasts were pre-treated with TGF β 1 for 48 h followed by IL1 β for 48 h and quantified by qRT-PCR and expressed as fold change compared to untreated control.
doi:10.1371/journal.pone.0091559.g009

IL1 β reduces TGF β 1-induced LOX mRNA expression and activity

A basic step in cross-link formation is the formation of aldehydes by means of lysyl oxidase (LOX). LOX is an extracellular enzyme involved in the intermolecular cross-linking between collagen molecules within fibrils, enhancing the stability of the ECM [55]. Increased LOX activity and expression has been found in e.g. skin and lung fibrosis [56,57,58]. As expected, the expression was significantly increased in TGF β 1-stimulated HDFa and HLFa (Fig. 10A–B). Interestingly, the presence of IL1 β resulted in a significant down-regulation of the TGF β 1-induced expression of LOX in either type of fibroblasts at 48 h (Fig. 10A–B). Fluorometric determination of LOX activity in the culture medium showed a positive correlation with mRNA data (Fig. 10C–D). Upon stimulation with IL1 β , no obvious change in LOX activity was seen for HDFa and HLFa, whereas activity was increased in TGF β 1-exposed fibroblasts after 48 h. A combination of IL1 β with TGF β 1 decreased the LOX activity in the culture medium of HDFa and HLFa at 48 h (Fig. 10C–D).

IL1 β activates expression of the mRNA of MMPs in TGF β 1-enriched fibroblasts

In fibrosis, a disbalance is seen between collagen expression and the expression of collagenases and gelatinases: the increase in collagen expression is not accompanied with an increase in MMP expression. We investigated the effect of IL1 β on the expression of MMP1, –2, –9 and –14 in a non-fibrotic (without TGF β 1) and in a pro-fibrotic (with TGF β 1) environment (Fig. 11–12). We observed that IL1 β resulted in a significant upregulation of MMP1, –2 and –14 in a non-fibrotic environment both in HDFa and HLFa. As expected, TGF β 1 alone did not upregulate gene expression of the measured MMPs. Stimulation of fibroblasts with IL1 β resulted in an upregulation of most MMPs compared to non-stimulated cells. The increased gene expression of MMPs after IL1 β stimulation was also obvious in the presence of TGF β 1 (Fig. 11A–H and Fig. 12A–D). However, TGF β 1 did affect the level of gene expression of certain MMPs in the presence of IL1 β . The increased expression of MMP14 caused by IL1 β was dampened by TGF β 1 in HLFa, whereas no such attenuation

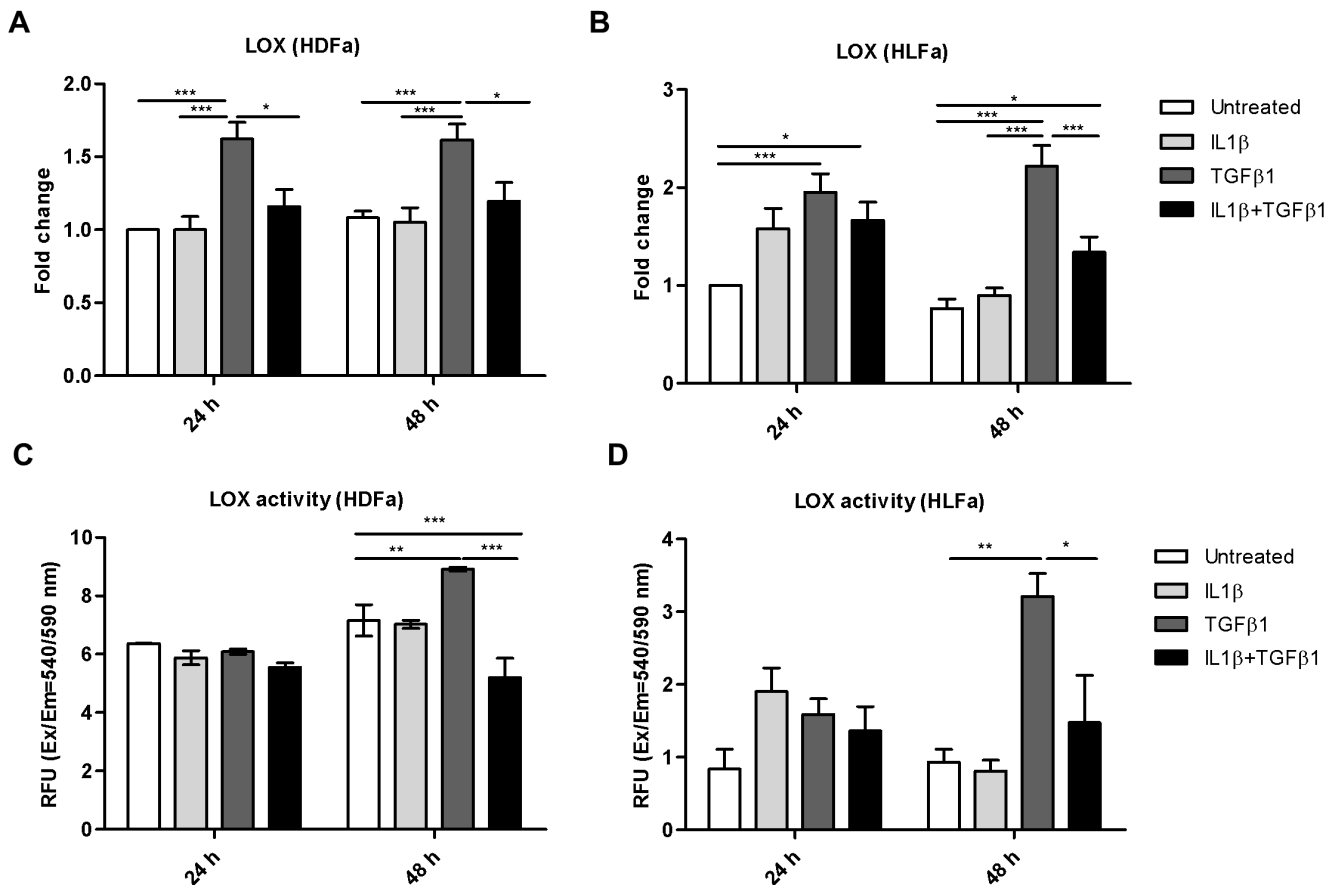


Figure 10. Effects of IL1 β and TGF β 1 on the expression and activity level of lysyl oxidase in dermal and lung fibroblasts. (A–B) HDFa and HLFa were treated with IL1 β , TGF β 1, or a combination of both, for 24 and 48 h. The mRNA levels of LOX were measured with qRT-PCR and expressed as fold change compared to untreated control. (C–D) Quantification of lysyl oxidase activity as secreted in the culture medium by HDFa and HLFa treated with IL1 β , TGF β 1, or a combination of both for 24 and 48 h.
doi:10.1371/journal.pone.0091559.g010

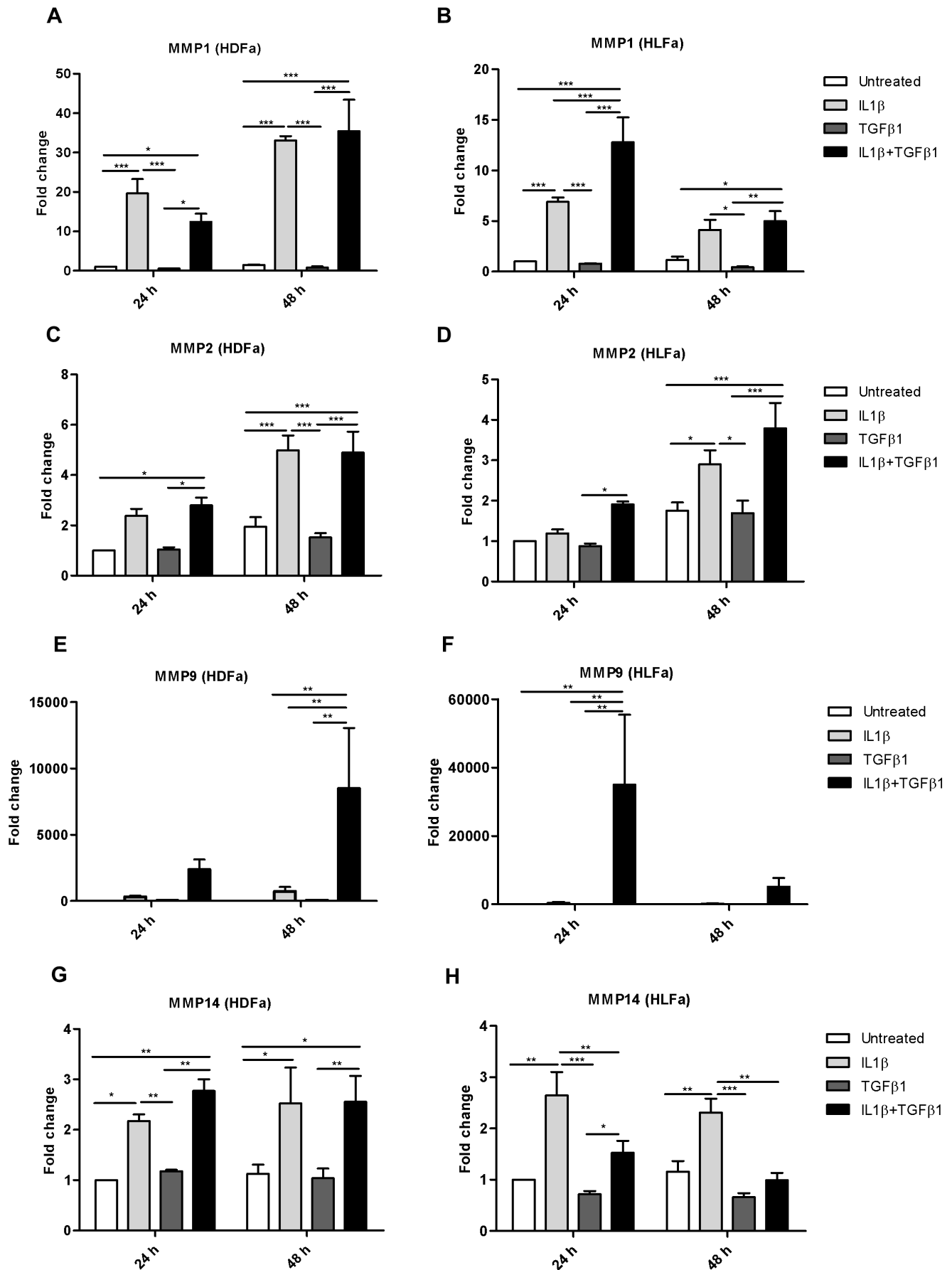


Figure 11. Effects of IL1 β and TGF β 1 on the expression level of matrix metalloproteinases in dermal and lung fibroblasts. HDFa and HLFa were treated with IL1 β , TGF β 1, or a combination of both, for 24 and 48 h. (A–H) The mRNA levels of MMP1, –2, –9 and 14 were measured with qRT-PCR and expressed as fold change compared to untreated control. doi:10.1371/journal.pone.0091559.g011

was seen in HDFa (Fig. 11G–H). In contrast, the increased MMP9 levels caused by IL1 β were even further upregulated in the presence of TGF β 1 (Fig. 11E–F). Fibroblasts treated with TGF β 1 for 48 h followed by IL1 β treatment for 48 h showed a high upregulation of MMP9 (Fig. 12A–B). Such an upregulation was also observed for MMP1 in HDFa, whereas a pre-treatment with TGF β 1 resulted in a dampening of MMP1 expression in HLFa (Fig. 12C–D).

Discussion

It has been reported, that IL1 β has a deleterious role in fibrosis *in vivo*. However, in these studies the role of IL1 β in the different phases of fibrosis, i.e. the acute inflammation phase and the later stages of inflammation and tissue repair, has not been dissected. Furthermore, relatively little is known about the direct effects of IL1 β on fibroblasts, and even less is known whether IL1 β influences the pro-fibrotic effect of TGF β 1 towards fibroblasts. We have investigated the direct role of IL1 β on dermal and lung fibroblasts, in the presence or absence of TGF β 1, and found that IL1 β alone did not contribute to the formation of myofibroblasts, and that IL1 β on the contrary is able to attenuate or even reverse the pro-fibrotic effects of TGF β 1.

Although IL1 β alone did show a stimulatory effect on the expression of COL3A1 and the collagen-modifying enzymes P4HA1, P4HB, PLOD1 and PLOD2, we observed that IL1 β alone had no stimulatory effect of GLI1, GLI1 Δ N, COL1A1,

ACTA2 and TAGLN mRNA expression levels (i.e. it did not contribute to the activation of fibroblasts into myofibroblasts or to the increase in collagen type I expression). Furthermore, IL1 β alone *did* have a stimulatory effect on the expression of collagenolytic enzymes MMP1, –2, –9 and –14, which is another potentially positive (i.e. anti-fibrotic) effect. A major finding of our study is further, that the co-presence of IL1 β inhibited the pro-fibrotic stimulatory effects of TGF β 1, by decreasing the mRNA levels of GLI1, GLI1 Δ N, COL1A1, ACTA2 and TAGLN, an effect that was verified on a protein level as well. Thus, IL1 β inhibits myofibroblast formation as induced by TGF β 1, and therefore potentially could attenuate the severity of fibrosis. Other potentially positive (i.e. anti-fibrotic) effects of IL1 β were the increased levels of MMP1, –2, –9 and –14 produced by fibroblasts exposed to TGF β 1/IL1 β compared to fibroblasts exposed to TGF β 1 alone. In addition, IL1 β decreased the TGF β 1-induced upregulation of LOX, which was verified by means of measuring total lysyl oxidase activity levels in the culture medium. Lysyl oxidase is involved in the cross-linking of collagen, making the collagen network more resistant towards proteolytic enzymes. Lowering lysyl oxidase activity levels has anti-fibrotic effects [58,59].

The mechanisms of IL1 β to attenuate the TGF β 1-induced myofibroblast formation are largely unknown. However, recent studies regarding the mechanism of fibroblast to myofibroblast transition has led to a renewed interest in the non-canonical activation of the sonic Hedgehog (SHH) pathway, especially with

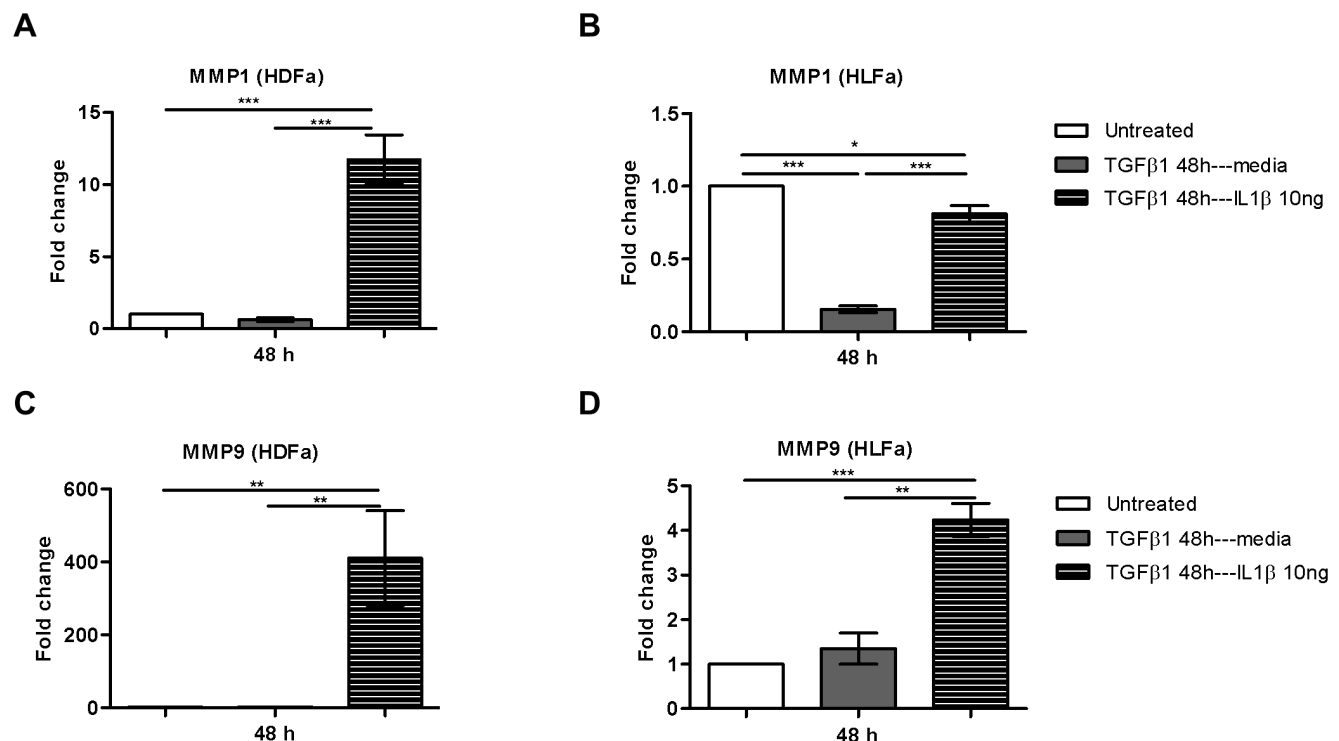


Figure 12. Post-treatment effects of IL1 β on TGF β 1-stimulated dermal and lung fibroblasts on the expression level of matrix metalloproteinases. Fibroblasts were pre-treated with TGF β 1 for 48 h followed by IL1 β for 48 h and quantified by qRT-PCR and expressed as fold change compared to untreated control. (A–B) The mRNA levels of MMP1 in HDFa and HLFa. (C–D) The mRNA levels of MMP9 in HDFa and HLFa. doi:10.1371/journal.pone.0091559.g012

respect to the transcription factor GLI1 [11,12,14]. It has been shown that the SHH pathway is activated in fibrotic conditions [60,61,62], that GLI1 expression is induced by TGF β 1, and the transformation of fibroblasts into myofibroblasts is GLI1-dependent [14,43]. In our study we also observed an upregulation of GLI1 by TGF β 1, but this was reduced by the addition of IL1 β , and as a consequence myofibroblast formation was hampered. A similar effect was seen for the isoform of GLI1, GLI1 Δ N. Although our study showed that regulation of GLI1 Δ N was similar to GLI1, the function of GLI1 Δ N needs to be further investigated. The ability of IL1 β to downregulate GLI1 could potentially be anti-fibrotic, as GLI activation was found to be required for TGF β 1-induced myofibroblast differentiation [14]. Inhibition of GLI by GANT61 (i.e. directly blocking the binding of GLI1 and GLI2 to their DNA targets) abrogated the effect of TGF β 1 on protein levels of α SMA and collagen type I in human lung fibroblasts [14]. As with fibroblasts, activation of the SHH pathway results in the transdifferentiation of hepatic stellate cells into myofibroblasts, a key mechanism in liver fibrosis [63,64,65,66]. Pharmacological inhibition of the SHH pathway results in an attenuation of fibrosis in a variety of *in vivo* models, namely unilateral ureteral obstruction (UUO) [67], systemic sclerosis [68] and liver cirrhosis [66,69,70], although others did not report improvements [71,72].

Another potential anti-fibrotic feature of IL1 β might be its ability to upregulate COL3A1. In all cases, stimulation with IL1 β resulted in an increased collagen type III to collagen type I ratio, as expression of collagen type I went down and that of collagen type III went up or remained the same. Interestingly, an increased collagen III:I ratio is often found in situations where a scarless healing is observed [73]. A prerequisite is that the produced collagens are adequately modified. As stimulation with IL1 β did not result in a decrease in the expression level of prolyl hydroxylase or lysyl hydroxylase, this seems to be the case.

A negative finding of our study was the steep increase in PLOD2 expression when IL1 β is added to TGF β 1. Increased levels of PLOD2 are seen in fibrotic conditions [49,50], resulting in the formation of increased levels of pyridinium cross-links *at the expense* of other cross-links, and making the collagen more difficult to degrade. However, this might be compensated with IL1 β by (1) inhibiting the TGF β 1-induced upregulation of LOX (resulting in less lysyl oxidase activity levels, which likely results in a decrease in the *total* amount of cross-links), and (2) by upregulating the expression of various MMPs, which likely results in a higher collagenolytic potential.

Our data might have major implications regarding the possible use of IL1 β inhibitors in fibrosis. We showed that IL1 β is able to suppress the pro-fibrotic features of TGF β 1, and thus shows potential anti-fibrotic properties, at least in the later stages of tissue repair, where TGF β levels are more prominent. It is possible that inhibition of IL1 β in the early stages of tissue repair alone has better effects than continuous suppression of IL1 β activity during the entire repair process. Inhibiting the early inflammatory cascade certainly has an anti-fibrotic effect, but inhibiting IL1 β at the later phases of the healing response might have a negative effect on the direct anti-fibrotic properties of IL1 β on fibroblasts in the presence of TGF β . In this context it is of interest, that a single injection of an IL1 β inhibitor immediately after ligation in a non-reperused infarction model attenuated the collagen accumulation in the infarcted area [74], indicating that the timing and duration of intervention with regard to IL1 β is a key determinant of

outcome [75]. Another relevant finding from our study is that fibroblasts from different origins (in our case lung and skin) do not always behave identical towards IL1 β . Most marked in this respect is the magnitude of COL1A1 suppression by IL1 β between dermal and lung fibroblasts after the pre-treatment with TGF β 1, and the magnitude of upregulation of COL3A1 and MMP1 by IL1 β after the pre-treatment with TGF β 1. This difference in behaviour between dermal and lung fibroblasts is in line with the observation (albeit widely neglected) that fibroblasts derived from different organs show major differences in phenotypic properties, as reflected by huge differences in gene expression patterns [37,38]. These phenotypical differences should be taken into account in the search for anti-fibrotic agents: an inhibitor may not have the desired effect in all organ systems due to the different response of the fibroblasts.

In conclusion we have shown that IL1 β counteracts a central process in fibrogenesis, namely the TGF β 1-mediated transition of fibroblasts into myofibroblasts. Clearly, the role of IL1 β in fibrosis should be reconsidered. Furthermore, we have shown that fibroblasts from different origin (i.e. lung and dermal fibroblasts) show different phenotypical responses towards IL1 β . Both these findings should be taken into consideration in the development of future anti-fibrotic therapies. In the future we aim to investigate what the underlying mechanism of IL1 β is to the actions of TGF β 1-induced pro-fibrotic responses in fibroblasts with respect to myofibroblast differentiation and collagen biosynthesis.

Supporting Information

Figure S1 Dose-dependent effects of TGF β 1 and IL1 β on the genes expression level of pro-fibrotic markers in dermal and lung fibroblasts. HDFa and HLFa were treated with TGF β 1 (0.5 and 2 ng/ml), IL1 β (10 ng/ml), a combination thereof, or both, for 48 hours (A–F). The mRNA levels of ACTA2, GLI1 and COL1A1 were quantified with qRT-PCR and expressed as a fold change compare to untreated control. Gene expression data was normalized to the reference gene YWHAZ. Data are represented as mean \pm SEM of quadruplicate experiments.

(TIF)

Figure S2 Concentration-dependent effects of TGF β 1 and IL1 β on the gene expression level of the collagen-modifying and -degrading enzymes in dermal and lung fibroblasts. HDFa and HLFa were treated with TGF β 1 (0.5 and 2 ng/ml), IL1 β (10 ng/ml), a combination thereof, or both, for 48 hours (A–F). The mRNA levels of PLOD2, MMP1 and MMP9 were quantified with qRT-PCR and expressed as a fold change compare to untreated control. Gene expression data was normalized to the reference gene YWHAZ. Data are represented as mean \pm SEM of quadruplicate experiments.

(TIF)

Methods S1 Cell culture and qRT-PCR.

(DOCX)

Author Contributions

Conceived and designed the experiments: MMM RAB. Performed the experiments: MMM. Analyzed the data: MMM. Contributed reagents/materials/analysis tools: MMM. Wrote the paper: MMM MB RAB. Interpreted the data and edited the manuscript: MB. Supervised the research work, interpreted the data, and edited the manuscript: RAB.

References

- Bujak M, Dobaczewski M, Chatila K, Mendoza LH, Li N, et al. (2008) Interleukin-1 receptor type I signaling critically regulates infarct healing and cardiac remodeling. *Am J Pathol* 173: 57–67.
- Gasse P, Mary C, Guenon I, Noulin N, Charron S, et al. (2007) IL-1R1/MyD88 signaling and the inflammasome are essential in pulmonary inflammation and fibrosis in mice. *J Clin Invest* 117: 3786–3799.
- Gieling RG, Wallace K, Han YP. (2009) Interleukin-1 participates in the progression from liver injury to fibrosis. *Am J Physiol Gastrointest Liver Physiol* 296: G1324–31.
- Guo J, Gu N, Chen J, Shi T, Zhou Y, et al. (2013) Neutralization of interleukin-1 beta attenuates silica-induced lung inflammation and fibrosis in C57BL/6 mice. *Arch Toxicol*.
- Kamari Y, Shaish A, Vax E, Shemesh S, Kandel-Kfir M, et al. (2011) Lack of interleukin-1alpha or interleukin-1beta inhibits transformation of steatosis to steatohepatitis and liver fibrosis in hypercholesterolemic mice. *J Hepatol* 55: 1086–1094.
- Kolb M, Margetts PJ, Anthony DC, Pitossi F, Gauldie J. (2001) Transient expression of IL-1beta induces acute lung injury and chronic repair leading to pulmonary fibrosis. *J Clin Invest* 107: 1529–1536.
- Wilson MS, Madala SK, Ramalingam TR, Gochuico BR, Rosas IO, et al. (2010) Bleomycin and IL-1beta-mediated pulmonary fibrosis is IL-17A dependent. *J Exp Med* 207: 535–552.
- Chaudhuri V, Zhou L, Karasek M. (2007) Inflammatory cytokines induce the transformation of human dermal microvascular endothelial cells into myofibroblasts: A potential role in skin fibrogenesis. *J Cutan Pathol* 34: 146–153.
- Gabbiani G. (2003) The myofibroblast in wound healing and fibrocontractive diseases. *J Pathol* 200: 500–503.
- Evans RA, Tian YC, Steadman R, Phillips AO. (2003) TGF-beta1-mediated fibroblast-myofibroblast terminal differentiation-the role of smad proteins. *Exp Cell Res* 282: 90–100.
- Bolanos AL, Milla CM, Lira JC, Ramirez R, Checa M, et al. (2012) Role of sonic hedgehog in idiopathic pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 303: L978–90.
- Horn A, Palumbo K, Cordazzo C, Dees C, Akhmetshina A, et al. (2012a) Hedgehog signaling controls fibroblast activation and tissue fibrosis in systemic sclerosis. *Arthritis Rheum* 64: 2724–2733.
- Wynn TA, Ramalingam TR. (2012) Mechanisms of fibrosis: Therapeutic translation for fibrotic disease. *Nat Med* 18: 1028–1040.
- Cigna N, Farrokhi Moshai E, Brayer S, Marchal-Somme J, Wemeau-Stervinou L, et al. (2012) The hedgehog system machinery controls transforming growth factor-beta-dependent myofibroblastic differentiation in humans: Involvement in idiopathic pulmonary fibrosis. *Am J Pathol* 181: 2126–2137.
- Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G. (1993) Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* 122: 103–111.
- Border WA, Noble NA. (1994) Transforming growth factor beta in tissue fibrosis. *N Engl J Med* 331: 1286–1292.
- Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J. (1997) Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. *J Clin Invest* 100: 768–776.
- Hinz B, Gabbiani G. (2003) Cell-matrix and cell-cell contacts of myofibroblasts: Role in connective tissue remodeling. *Thromb Haemost* 90: 993–1002.
- Ishida W, Mori Y, Lakos G, Sun L, Shan F, et al. (2006) Intracellular TGF-beta receptor blockade abrogates smad-dependent fibroblast activation in vitro and in vivo. *J Invest Dermatol* 126: 1733–1744.
- Usuki J, Matsuda K, Azuma A, Kudoh S, Gemma A. (2012) Sequential analysis of myofibroblast differentiation and transforming growth factor-beta1/Smad pathway activation in murine pulmonary fibrosis. *J Nippon Med Sch* 79: 46–59.
- Mirastschijski U, Schnabel R, Claes J, Schneider W, Agren MS, et al. (2010) Matrix metalloproteinase inhibition delays wound healing and blocks the latent transforming growth factor-beta1-promoted myofibroblast formation and function. *Wound Repair Regen* 18: 223–234.
- Branski RC, Perera P, Verdolini K, Rosen CA, Hebda PA, et al. (2007) Dynamic biomechanical strain inhibits IL-1beta-induced inflammation in vocal fold fibroblasts. *J Voice* 21: 651–660.
- Furuyama A, Hosokawa T, Mochitate K. (2008) Interleukin-1beta and tumor necrosis factor-alpha have opposite effects on fibroblasts and epithelial cells during basement membrane formation. *Matrix Biol* 27: 429–440.
- Honda A, Abe R, Makino T, Norisugi O, Fujita Y, et al. (2008) Interleukin-1beta and macrophage migration inhibitory factor (MIF) in dermal fibroblasts mediate UVA-induced matrix metalloproteinase-1 expression. *J Dermatol Sci* 49: 63–72.
- Moon DO, Kim MO, Choi YH, Park YM, Kim GY. (2010) Curcumin attenuates inflammatory response in IL-1beta-induced human synovial fibroblasts and collagen-induced arthritis in mouse model. *Int Immunopharmacol* 10: 605–610.
- Siwik DA, Chang DL, Colucci WS. (2000) Interleukin-1beta and tumor necrosis factor-alpha decrease collagen synthesis and increase matrix metalloproteinase activity in cardiac fibroblasts in vitro. *Circ Res* 86: 1259–1265.
- Thampatty BP, Li H, Im HJ, Wang JH. (2007) EP4 receptor regulates collagen type-I, MMP-1, and MMP-3 gene expression in human tendon fibroblasts in response to IL-1 beta treatment. *Gene* 386: 154–161.
- Xiao H, Ji AM, Li ZL, Song XD, Su D, et al. (2008) Interleukin-1beta inhibits collagen synthesis and promotes its decomposition in cultured cardiac fibroblasts. *Sheng Li Xue Bao* 60: 355–361.
- Barkhordar RA, Ghani QP, Russell TR, Hussain MZ. (2002) Interleukin-1beta activity and collagen synthesis in human dental pulp fibroblasts. *J Endod* 28: 157–159.
- Lu HK, Tseng CC, Lee YH, Li CL, Wang LF. (2010) Flutamide inhibits nifedipine- and interleukin-1 beta-induced collagen overproduction in gingival fibroblasts. *J Periodontol Res* 45: 451–457.
- Vesey DA, Cheung C, Cuttle L, Endre Z, Gobe G, et al. (2002) Interleukin-1beta stimulates human renal fibroblast proliferation and matrix protein production by means of a transforming growth factor-beta-dependent mechanism. *J Lab Clin Med* 140: 342–350.
- Heino J, Heimonen T. (1990) Interleukin-1 beta prevents the stimulatory effect of transforming growth factor-beta on collagen gene expression in human skin fibroblasts. *Biochem J* 271: 827–830.
- Hong HH, Trackman PC. (2002) Cytokine regulation of gingival fibroblast lysyl oxidase, collagen, and elastin. *J Periodontol* 73: 145–152.
- Lawrance IC, Maxwell L, Doe W. (2001) Altered response of intestinal mucosal fibroblasts to profibrogenic cytokines in inflammatory bowel disease. *Inflamm Bowel Dis* 7: 226–236.
- Vesey DA, Cheung CW, Cuttle L, Endre ZA, Gobe G, et al. (2002) Interleukin-1beta induces human proximal tubule cell injury, alpha-smooth muscle actin expression and fibronectin production. *Kidney Int* 62: 31–40.
- Maleszewska M, Moonen JR, Huijman N, van de Sluis B, Krenning G, et al. (2013) IL-1beta and TGFbeta2 synergistically induce endothelial to mesenchymal transition in an NFkappaB-dependent manner. *Immunobiology* 218: 443–454.
- Chang HY, Chi JT, Dudoit S, Bondre C, van de Rijn M, et al. (2002) Diversity, topographic differentiation, and positional memory in human fibroblasts. *Proc Natl Acad Sci U S A* 99: 12877–12882.
- Rinn JL, Bondre C, Gladstone HB, Brown PO, Chang HY. (2006) Anatomic demarcation by positional variation in fibroblast gene expression programs. *PLoS Genet* 2: e119.
- Broekelmann TJ, Limper AH, Colby TV, McDonald JA. (1991) Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. *Proc Natl Acad Sci U S A* 88: 6642–6646.
- Lazard D, Sastre X, Frid MG, Glukhova MA, Thierry JP, et al. (1993) Expression of smooth muscle-specific proteins in myoepithelium and stromal myofibroblasts of normal and malignant human breast tissue. *Proc Natl Acad Sci U S A* 90: 999–1003.
- Gabbiani G. (1994) Modulation of fibroblastic cytoskeletal features during wound healing and fibrosis. *Pathol Res Pract* 190: 851–853.
- Desmouliere A, Gabbiani G. (1994) Modulation of fibroblastic cytoskeletal features during pathological situations: The role of extracellular matrix and cytokines. *Cell Motil Cytoskeleton* 29: 195–203.
- Dennler S, Andre J, Alexaki I, Li A, Magaldi T, et al. (2007) Induction of sonic hedgehog mediators by transforming growth factor-beta: Smad3-dependent activation of Gli2 and Gli1 expression in vitro and in vivo. *Cancer Res* 67: 6981–6986.
- Shimokawa T, Tostar U, Lauth M, Palaniswamy R, Kasper M, et al. (2008) Novel human glioma-associated oncogene 1 (GLI1) splice variants reveal distinct mechanisms in the terminal transduction of the hedgehog signal. *J Biol Chem* 283: 14345–14354.
- Mori Y, Ishida W, Bhattacharyya S, Li Y, Platanias LC, et al. (2004) Selective inhibition of activin receptor-like kinase 5 signaling blocks profibrotic transforming growth factor beta responses in skin fibroblasts. *Arthritis Rheum* 50: 4008–4021.
- Nakayama S, Mukae H, Sakamoto N, Kakugawa T, Yoshioka S, et al. (2008) Pirfenidone inhibits the expression of HSP47 in TGF-beta1-stimulated human lung fibroblasts. *Life Sci* 82: 210–217.
- Hisatomi K, Mukae H, Sakamoto N, Ishimatsu Y, Kakugawa T, et al. (2012) Pirfenidone inhibits TGF-beta1-induced over-expression of collagen type I and heat shock protein 47 in A549 cells. *BMC Pulm Med* 12: 24–2466–12–24.
- Poole A, Myllyla R, Wagner JC, Brown RC. (1985) Collagen biosynthesis enzymes in lung tissue and serum of rats with experimental silicosis. *Br J Exp Pathol* 66: 567–575.
- van der Slot AJ, Zuurmond AM, Bardoe AF, Wijnga C, Pruijs HE, et al. (2003) Identification of PLOD2 as telopeptide lysyl hydroxylase, an important enzyme in fibrosis. *J Biol Chem* 278: 40967–40972.
- van der Slot AJ, Zuurmond AM, van den Bogaert AJ, Ulrich MM, Middelkoop E, et al. (2004) Increased formation of pyridinoline cross-links due to higher telopeptide lysyl hydroxylase levels is a general fibrotic phenomenon. *Matrix Biol* 23: 251–257.
- Seth P, Yeowell HN. (2010) Fox-2 protein regulates the alternative splicing of scleroderma-associated lysyl hydroxylase 2 messenger RNA. *Arthritis Rheum* 62: 1167–1175.

52. van der Slot AJ, van Dura EA, de Wit EC, De Groot J, Huizinga TW, et al. (2005) Elevated formation of pyridinoline cross-links by profibrotic cytokines is associated with enhanced lysyl hydroxylase 2b levels. *Biochim Biophys Acta* 1741: 95–102.
53. van der Slot-Verhoeven AJ, van Dura EA, Attema J, Blauw B, Degroot J, et al. (2005) The type of collagen cross-link determines the reversibility of experimental skin fibrosis. *Biochim Biophys Acta* 1740: 60–67.
54. Brinckmann J, Kim S, Wu J, Reinhardt DP, Batmunkh C, et al. (2005) Interleukin 4 and prolonged hypoxia induce a higher gene expression of lysyl hydroxylase 2 and an altered cross-link pattern: Important pathogenetic steps in early and late stage of systemic scleroderma? *Matrix Biol* 24: 459–468.
55. Trackman PC. (2005) Diverse biological functions of extracellular collagen processing enzymes. *J Cell Biochem* 96: 927–937.
56. Szauder KM, Cao T, Boyd CD, Csiszar K. (2005) Lysyl oxidase in development, aging and pathologies of the skin. *Pathol Biol (Paris)* 53: 448–456.
57. Counts DF, Evans JN, Dipetrillo TA, Sterling KM Jr, Kelley J. (1981) Collagen lysyl oxidase activity in the lung increases during bleomycin-induced lung fibrosis. *J Pharmacol Exp Ther* 219: 675–678.
58. Li S, Yang X, Li W, Li J, Su X, et al. (2012) N-acetylcysteine downregulation of lysyl oxidase activity alleviating bleomycin-induced pulmonary fibrosis in rats. *Respiration* 84: 509–517.
59. Kagan HM. (2000) Intra- and extracellular enzymes of collagen biosynthesis as biological and chemical targets in the control of fibrosis. *Acta Trop* 77: 147–152.
60. Guy CD, Suzuki A, Zdanowicz M, Abdelmalek MF, Burchette J, et al. (2012) Hedgehog pathway activation parallels histologic severity of injury and fibrosis in human nonalcoholic fatty liver disease. *Hepatology* 55: 1711–1721.
61. Michelotti GA, Xie G, Swiderska M, Choi SS, Karaca G, et al. (2013) Smoothened is a master regulator of adult liver repair. *J Clin Invest* 123: 2380–2394.
62. Swiderska-Syn M, Suzuki A, Guy CD, Schwimmer JB, Abdelmalek MF, et al. (2013) Hedgehog pathway and pediatric nonalcoholic fatty liver disease. *Hepatology* 57: 1814–1825.
63. Choi SS, Omenetti A, Witek RP, Moylan CA, Syn WK, et al. (2009) Hedgehog pathway activation and epithelial-to-mesenchymal transitions during myofibroblastic transformation of rat hepatic cells in culture and cirrhosis. *Am J Physiol Gastrointest Liver Physiol* 297: G1093–106.
64. Choi SS, Syn WK, Karaca GF, Omenetti A, Moylan CA, et al. (2010) Leptin promotes the myofibroblastic phenotype in hepatic stellate cells by activating the hedgehog pathway. *J Biol Chem* 285: 36551–36560.
65. Choi SS, Omenetti A, Syn WK, Diehl AM. (2011) The role of hedgehog signaling in fibrogenic liver repair. *Int J Biochem Cell Biol* 43: 238–244.
66. Chen Y, Choi SS, Michelotti GA, Chan IS, Swiderska-Syn M, et al. (2012) Hedgehog controls hepatic stellate cell fate by regulating metabolism. *Gastroenterology* 143: 1319–29.e1–11.
67. Ding H, Zhou D, Hao S, Zhou L, He W, et al. (2012) Sonic hedgehog signaling mediates epithelial-mesenchymal communication and promotes renal fibrosis. *J Am Soc Nephrol* 23: 801–813.
68. Horn A, Kireva T, Palumbo-Zerr K, Dees C, Tomcik M, et al. (2012) Inhibition of hedgehog signalling prevents experimental fibrosis and induces regression of established fibrosis. *Ann Rheum Dis* 71: 785–789.
69. Philips GM, Chan IS, Swiderska M, Schroder VT, Guy C, et al. (2011) Hedgehog signaling antagonist promotes regression of both liver fibrosis and hepatocellular carcinoma in a murine model of primary liver cancer. *PLoS One* 6: e23943.
70. Pratap A, Singh S, Munda V, Yang N, Panakanti R, et al. (2012) Attenuation of early liver fibrosis by pharmacological inhibition of smoothened receptor signaling. *J Drug Target* 20: 770–782.
71. Fabian SL, Penchev RR, St-Jacques B, Rao AN, Sipila P, et al. (2012) Hedgehog-gli pathway activation during kidney fibrosis. *Am J Pathol* 180: 1441–1453.
72. Liu L, Kugler MC, Loomis CA, Samdani R, Zhao Z, et al. (2013) Hedgehog signaling in neonatal and adult lung. *Am J Respir Cell Mol Biol* 48: 703–710.
73. Namazi MR, Fallahzadeh MK, Schwartz RA. (2011) Strategies for prevention of scars: What can we learn from fetal skin? *Int J Dermatol* 50: 85–93.
74. Hwang MW, Matsumori A, Furukawa Y, Ono K, Okada M, et al. (2001) Neutralization of interleukin-1 β in the acute phase of myocardial infarction promotes the progression of left ventricular remodeling. *J Am Coll Cardiol* 38: 1546–1553.
75. Bujak M, Frangogiannis NG. (2009) The role of IL-1 in the pathogenesis of heart disease. *Arch Immunol Ther Exp (Warsz)* 57: 165–176.